

FORM PTO-1390 (Modified)
(REV. 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES

71745/55880

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

09/830980

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/IB99/01891

3 November 1999

3 November 1998

TITLE OF INVENTION

REGULATOR OF NOTCH SIGNALING ACTIVITY

APPLICANT(S) FOR DO/EO/US

Stephen COHEN, Antonius BOUWMEESTER, Julien ROYET

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Published PCT Application WO 00/26364

Copy of Form PCT/ISA/210

Copy of Form PCT/IPEA/401

Copy of Form PCT/RO/101

Copy of Form PCT/IB/308

Copy of Form PCT/IPEA/416

Copy of Form PCT/IB/304

Copy of Form PCT/IPEA/402

Substitute Sequence Listing 2 pgs.

Preliminary Amendment. and Transmittal of Formal Drawing with FIG. 1 and marked-up FIG. 1

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/830980)		INTERNATIONAL APPLICATION NO. PCT/IB99/01891		ATTORNEY'S DOCKET NUMBER 71745/55880	
24. The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :					
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO				\$1000.00	
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO				\$860.00	
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO				\$710.00	
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)				\$690.00	
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)				\$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	25 - 20 =	5	x \$18.00	\$90.00	
Independent claims	3 - 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable).			<input checked="" type="checkbox"/>	\$270.00	
TOTAL OF ABOVE CALCULATIONS =				\$1,350.00	
<input type="checkbox"/> Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				\$0.00	
SUBTOTAL =				\$1,350.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				\$0.00	
TOTAL NATIONAL FEE =				\$1,350.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).			<input type="checkbox"/>	\$0.00	
TOTAL FEES ENCLOSED =				\$1,350.00	
				Amount to be: refunded	\$
				charged	\$

- a. ☒ A check in the amount of **\$1,350.00** to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **04-1105** A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

Cara Z. Lowen

NAME

38,227

REGISTRATION NUMBER

May 2, 2001

DATE

09/830980
JC08 Rec'd PCT/PTO 02 MAY 2001

Attorney Docket No. 71745 (55880)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): Cohen et al.

EXAMINER: N/A

U.S.S.N.: UNASSIGNED (National Application based on
PCT/IB99/01891)

GROUP: N/A

FILED: HERewith

FOR: REGULATOR OF NOTCH SIGNALING ACTIVITY

Box Patent Application
Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service, in an envelope as "Express Mail Post Office Addressee" Mailing Label Number EL 730723823 US addressed to BOX PATENT APPLICATION the Commission of Patents, Washington, D.C. 20231 on May 2, 2001.

By


JUDITH A. HERRICK

PRELIMINARY AMENDMENT AND TRANSMITTAL OF FORMAL DRAWING

Sir:

Prior to examination, please make the following amendments to the above-referenced patent application:

IN THE FIGURES:

Please amend Figure 1 as indicated in the Figure attached hereto which shows the changes.

IN THE SEQUENCES:

Please replace pages 1 and 2 of the sequence listing with pages 1 and 2 of the sequence listing attached hereto.

Attorney Docket No. 71745 (55880)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): Cohen et al. EXAMINER: Unassigned
U.S.S.N.: 09/830,980 GROUP: Unassigned
FILED: May 2, 2001
Int. Appl. No.: PCT/IB99/01891
Priority Date: November 3, 1998
FOR: REGULATOR OF NOTCH SIGNALING ACTIVITY

CERTIFICATE OF MAILING

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service with sufficient postage as express mail (Express Mail Label No. EL933047787US in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 on January 14, 2002.

By: 

Donna M. Tomaso

Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Please amend the application as follows.

IN THE SPECIFICATION:

Please replace the paragraph at page 16, lines 11-22 with the following rewritten paragraph:

--A 15Kb *Sall* genomic fragment of phage Y2-6 was inserted into the *XhoI* site of the transformation vector pCaSpeR4. UAS-*Nle* was prepared by closing the 1.5Kb *Nle* cDNA as a *NotI*-*XhoI* fragment into pUAST (Brand and Perrimon, 1993). An HA-tagged

version of Nle was generated by introducing three copies of the HA epitope (YPUDVPDYA) (SEQ ID NO: 8) immediately downstream of the first Methionine residue. The *Bam*HI-*Ascl* fragment of pKS-Nle was replaced by a corresponding PCR fragment amplified using the following primers:

5' CGGATCCAAA AAATGTATCC CTATGACGTC CCCGATTATG CCTACCCTTA
CGATGTACCT GACTACGCGT ATCCGTACGA CGTTCCGGAC TATGCTCAGG
AGACGGACA CGGAGCAAGA GGCCACGCCA CATACGATAC AGGCGCGCCA A 3' (SEQ
ID NO: 9), and

5' TAAACGAGGC GCGCCTATCG TAT 3' (SEQ ID NO: 10).--

Please replace the paragraph at page 22, lines 18-22 with the following rewritten paragraph:

--*XNle* was isolated by PCR using the degenerate primers, F 5'-CGC AGA ATT
CCI TTY GAY GTI CCI GTI GAY AT-3' (SEQ ID NO: 11)
and R 5'-GGT GCT CGA GCY TGI GGY TGR TAI ATD ATR TC-3' (SEQ ID NO: 12),
designed against conserved peptides, PFDVPVDI (SEQ ID NO: 13) and DIIYQPQ (SEQ
ID NO: 14) respectively, found in the Nle domain of the vertebrate proteins identified
as expressed sequence tags.--

Please replace the paragraph at page 22, line 23 to page 23, line 2 with the following rewritten paragraph:

-- Phage stock of a stage 30 library (Stratagene) was used as template to amplify a 200bp fragment that spans Nle domain. Five independent clones were sequenced and found to be identical. This fragment was used to screen the stage 30 library, which resulted in the isolation of 25 positive clones of which the longest of 2.2Kb was sequenced on both strands. Temporal expression was assayed by RT-PCR analysis as

described by Bouwmeester *et al.*, 1996 using the following primer set that amplifies a XNle fragment of 135 bp; F 5'-CAC CAG ATA AAC TGC AGT TAG-3' (SEQ ID NO: 15), R 5'-CTG TTT CAA CTG ATT GCT TCT-3' (SEQ ID NO: 16) (28 cycles).--

Please substitute the attached sequence listing for the sequence listing in the application as filed and in the preliminary amendment filed on May 2, 2001.

REMARKS

Applicants submit herewith Sequence Listing substitute pages 1-12 to include as a Sequence Listing as part of this Application.

Applications have amended the Application to include the sequence identification number for SEQ ID Nos. 8-16 in the specification where reference is made to the sequence by use of the assigned identifier as required by 37 CFR §1.812(d). No new matter has been added by virtue of the amendment made to the specification.

Further enclosed is a computer readable copy of the above-mentioned copy of the Sequence Listing. That copy is the same as the copy of the Sequence listing.

Also enclosed is a Statement in Support of Filing and Submissions in Accordance with 37 CFR 1.821-1.825, which declares that the content of the paper and the computer readable copies of the Sequence Listing submitted in accordance with 37 CFR 1.821 (c) and (e), respectively, are the same and that the submission, filed in accordance with 37 CFR 1.821 (g).

S. Cohen, et al.
USSN: 09/830,980
Preliminary Amendment
Page -4-

STATEMENT UNDER 37 CFR §1.825(a)

I hereby state that the substitute sheets, which include the Sequence Listing are supported in the Application as filed. I hereby state that the substitute sheets do not include new matter.

The Commissioner is hereby authorized to charge any fees which may be required to consider this submission to Deposit Account No. **04-1105**.

Respectfully submitted,

Date: January 14, 2002

BOS2_177679.1

By: 

Cara Z. Lowen (Reg. No. 38,227)
Dike, Bronstein, Roberts & Cushman
Intellectual Property Practice Group
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Telephone: (617) 439-4444

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

The paragraph at page 16, lines 11-22 has been amended as follows:

A 15Kb *Sall* genomic fragment of phage Y2-6 was inserted into the *XhoI* site of the transformation vector pCaSpeR4. UAS-*Nle* was prepared by closing the 1.5Kb *Nle* cDNA as a *NotI*-*XhoI* fragment into pUAST (Brand and Perrimon, 1993). An HA-tagged version of *Nle* was generated by introducing three copies of the HA epitope (YPUDVPDYA) (SEQ ID NO: 8) immediately downstream of the first Methionine residue. The *Bam*HI-*Ascl* fragment of pKS-*Nle* was replaced by a corresponding PCR fragment amplified using the following primers:

5' CGGATCCAAA AAATGTATCC CTATGACGTC CCCGATTATG CCTACCCTTA
CGATGTACCT GACTACGCGT ATCCGTACGA CGTTCCGGAC TATGCTCAGG
AGACGGACA CGGAGCAAGA GGCCACGCCA CATACGATAC AGGCGCGCCA A 3' (SEQ
ID NO: 9), and

5' TAAACGAGGC GCGCCTATCG TAT 3' (SEQ ID NO: 10).

The paragraph at page 22, lines 18-22 has been amended as follows:

XNle was isolated by PCR using the degenerate primers, F 5'-CGC AGA ATT CCI
TTY GAY GTI CCI GTI GAY AT-3' (SEQ ID NO: 11) and R 5'-GGT GCT CGA GCY TGI
GGY TGR TAI ATD ATR TC-3' (SEQ ID NO: 12), designed against conserved peptides,
PFDVPVDI (SEQ ID NO: 13) and DIIYQPQ (SEQ ID NO: 14) respectively, found in the
Nle domain of the vertebrate proteins identified as expressed sequence tags.

The paragraph at page 22, line 23 to page 23, line 2 has been amended as follows:

Phage stock of a stage 30 library (Stratagene) was used as template to amplify a 200bp fragment that spans Nle domain. Five independent clones were sequenced and found to be identical. This fragment was used to screen the stage 30 library, which resulted in the isolation of 25 positive clones of which the longest of 2.2Kb was sequenced on both strands. Temporal expression was assayed by RT-PCR analysis as described by Bouwmeester *et al.*, 1996 using the following primer set that amplifies a XNle fragment of 135 bp; F 5'-CAC CAG ATA AAC TGC AGT TAG-3' (SEQ ID NO: 15), R 5'-CTG TTT CAA CTG ATT GCT TCT-3' (SEQ ID NO: 16) (28 cycles).

The attached sequence listing has been substituted for the sequence listing in the application as filed and in the preliminary amendment filed on May 2, 2001.

REMARKS

Enclosed please find Figure 1 consisting of 1 (one) sheet marked in red to identify the changes thereto. A discussion describing the revisions and the support therefore in the originally filed disclosure is provided below. Also enclosed please find Figure 1 consisting of 1 (one) sheet of the formal drawing for the subject application. In accordance with 37 C.F.R. 1.84(c), identifying indicia are provided on the backside of the sheet.

Applicants respectfully request that, prior to examination, Figure 1 be amended as shown on the marked-up version of Figure 1 and that pages 1 and 2 of the sequence listing be substituted with pages 1 and 2 submitted herewith. Applicants respectfully submit that no new matter is being added by the amendment of this figure and substitute sequence listing.

Figure 1 was originally filed with 9 (nine) residues inadvertently deleted from each row on the right hand side of the figure as indicated in the marked-up version submitted herewith. New Figure 1 sets forth the complete sequence for each organism. Applicants respectfully submit that, with the exception of the *Drosophila* ("fly") sequence, all the sequences in Figure 1 are known in the prior art, as described with genome database citations on page 3, lines 11-14 of the specification. Thus, one of ordinary skill in the pertinent art would have been able to obtain the correct sequences. In addition, the inadvertently omitted residues from the *Drosophila* sequence are described in the originally filed sequence listing in Sequence I.D No.1. Thus, no new matter has been added by virtue of this amendment.

Applicants further request that pages 1 and 2 of the sequence listing be replaced with the substituted pages submitted herewith. The Sequence I.D. No.1 in the original sequence listing was filed with 1 residue (K) inadvertently omitted in the second line of the listing (EIKSLED in the original should read EIK**K**SLED). This portion of the sequence was given correctly in the second line of the *Drosophila* ("fly") sequence in Figure 1 as originally filed and was also correctly shown in Figure 6B as originally filed. In addition, Sequence I.D. No.2 in the original sequence listing shows the DNA sequence encoding the correct protein, enabling one of ordinary skill in the pertinent art to obtain the correct sequence. Therefore, Applicants respectfully submit that no new matter is being added by the amendment of this sequence listing.

Should the Examiner wish to discuss the above amendment, the undersigned attorney would appreciate the opportunity to do so.

Applicants believe that additional fees are not required for consideration of the within Preliminary Amendment. However, if for any reason a fee is required, a fee paid is inadequate or credit is owed for any excess fee paid, you are hereby authorized and requested to charge Deposit Account No. **04-1105**.

Respectfully submitted,

Date:

5/2/01

By:

Cara Z. Lowen

Cara Z. Lowen (Reg. No. 38,227)
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Facsimile: (617) 523-6440

BOS2_167185.1

Omitted
in
original

Figure 1: multiple sequence alignment

```

yeast      MSTLIPPPSKKQKKEAQLPREVAII PKDLPNVSIKFQALDTGDNVGGALRVPGAISEKQL
c.elegans  -----PQISVSEDENELG---GSGILVPVDISTNEL
fly        -----MQETDTEQEATPHTIQARLVYTGEEAGPPIDLPAGITTTQQL
mouse      -----EEAAAGD-VQRLLVQFQDEGGQLLGSPFDVPVDITPDKL
human      -----GSPFDVPVDITPDRL
frog       -----MKEDVGRLLIQFKNENGEGLGTPFDVPLDITPDKL
           * : :* *: ..*

yeast      EELLNQLNGTSDDPVPYTFSCITQGGKASDPVKTIDITDNLVSSLIKPGYNSTEDQITLL
c.elegans  QILCNQLLGSR---FCLNNEFSVSG-----AEIVDSIRKSLEEIDFET---LKL
fly        GLICNALLKNE---EATPYLFFVGE-----DEIKKSLEDTLDLASVDI-ENVIDIV
mouse      XLVCNALL-AQEEPLPLAFYVHD-----AEIVSSLGKTLESQSV-ETEKIVDII
human      QLVCNALL-AQEDPCPLAFFVHD-----AEIVSSLGKTLESQAV-ETEKVLDIY
frog       QLVCNALL-QEEDPVPLAFFVQD-----LEIVTSLDKTLEKQSV-ETEKVIDII
           : * * : * .: .:* : :

yeast      YTPRAVFKVKPVTRSSSAIAGHGSTILCSAFAPHTSSRMVTGAGDNTARIWDCDTQTPMH
c.elegans  YQPQAVFRVRPVTRCSASIPGHGEPVISAQFSPDGRG-LASGSGDQTMRIWDIELELPLH
fly        YQPQAVFKVRPVTRCTSSMPGHAEAVVSLNFSPDGAH-LASGSGDTTVRLWDLNTETPHF
mouse      YQPQAVFRVRAVTRCTSS-----
human      -QPQXLFRVRAVTRCTS-----
frog       YQPQAVFKVRAVTRCTSSLEGHTEAVISVAFSPTGKY-LASGSGDTTVRFWDLSTETPHF
           *: :*: :*.***.: :

yeast      TLKGHYNWVLCVSWSPDGEVIATGSMNTIRLWDPKSGQCLGDALRGHSHKWTSLSWEPI
c.elegans  TCKSHKSWVLCIAWSPDATKIASACKNGEICIWNAKTGEQIGKTLKRHKQWIXXLAWQP-
fly        TCTGHKQWVLCVSWAPDGKRLASGCKAGSII IWDPETGQKGRPLSGHKKHINCLAWEPY
mouse      -----
human      -----
frog       TSKGHTHWVLSIAWSPDGKKLASGCKNSQIFIWDPSTGKQIGKPLTGHSKWITWLCWEPL

yeast      LVKPGSKPRLASSSKDGTIKIWDTVSRVCQYTMSGHTNSVSCVKWGGQGLLYSGSHDRTV
c.elegans  -----TVKMWR-----ADDGVMCRNMTG-----
fly        HRDPECR-KLASASGDGDCRIWDVKLGQCLMNIAGHTNAVTAVRWGGAGLIYTSSKDRTV
mouse      -----
human      -----
frog       HLNPESTRY-LASASKDCTIRIWDTVMGQCQKILTSHTQSVTAVKWGGDGLLYSSSQDRTI

yeast      RVWDINSQGRGINILKSHAHVWNHLSLSTDYALRIGAFDHTGKK-----PSTPEEAQKKA
c.elegans  -----HAHWINTLALNTDYALRTSCFE-----PSK-----
fly        KMWR-AADGILCRTFSGHAHWVNNIALSTDYVLRGTGPFHPVKDRSKSHLSLSTEELQESA
mouse      -----
human      -----
frog       KAWR-AQDGVLCRTLQGHAWVNTMALSTDYVLRKGAFNPADAS--VNPQDMSGSLLEVLR

yeast      LENYEKICKKNGNSEEMMVTASDDYTMFLWNPLKSTKPIARMTGHQKLVNHVAFSPDGRY
c.elegans  -----INRMTGHMQLVNOVVFPDTRY
fly        LKRYQAVCP---DEVESLVSCSDDNTLYLWRN-NQNKCVERTMTGHQNVVNDVKYSPDVKL
mouse      -----
human      -----
frog       EKALKRSNEVRGQGPRLVSGSEDFTLFLWAPAEKKPLQRMTHQALINEVLFSPDTRI

yeast      IVSASFNSIKLWDGRDGKFISTFRGHASVYQVAWSSDCRLLVSCSKDTTLKVWDVTRR
c.elegans  IASASFDSVKLWCGRTGKYLASFGRHVGPVYQVAWSADSRLLVSGSADSTLKVFEKTK
fly        IASASFDSVKLWRASDGQYMATFRGHVQAVYTVAWSADSRLIVSGSKDSTLKVWSVQTK
mouse      -----
human      -----
frog       IASASFDSIKLWDGKTGKFLTSLRGHVS AVYQIAWSADSRLLVSGSSDSTLKVWDSKTK

yeast      KLSVDLPGIKTKLY-VDWSVDGKRVCSSGKDKMVRLWTH
c.elegans  SLYYDLPGHGDVFTVDWSPEGTKVSGGKDKVLKLW--
fly        KLAQELPGHADEVFGVDWAPDGSRVASGGKDKVIKLWAY
mouse      -----
human      -----
frog       KLLIDLPGHADEVYSVDWSPDGQRVASGGKDKCLRIWRK

```

Omitted
in
original

Sequence I.D. No.1: Drosophila Notchless protein

MQETDTEQEATPHTIQARLVYTGEEAGPPIDLPA GITTQQLGLICNALLKNEEA
TPYLFFVGEDEIKKSLEDTLDLASVDTENVIDIVYQPQAVFKVRPVTRCTSSMP
5 GHAEAVVSLNFS PDGAHLASGSGD TT VRLWDLNTETPHFTCTGHKQWVLCV
SWAPDGKRLASGCKAGSIIIWDPETGQQKGRPLSGHKKHINCLAWEPYHRDP
ECRKLASASGDGDCRIWDVKLGQCLMNIAGHTNAVTAVRWGGAGLIYTSSK
DRTVKMWRAADGILCRTFSGHAHWVNNIALSTDYVLRTGPFHPVKDRSKSH
LSLSTEELQESALKRYQAVCPDEVESLVSCSDDNTLYLWRNNQNKCVERT
10 GHQNVVNDVKYSPDVKLIASASFDKSVRLWRASDGQYMATFRGHVQAVYT
VAWSADSR LIVSGSKDSTLKVWSVQTKKLAQELPGHADEVFGVDWAPDGSR
VASGGKDKVIKLWAY

Sequence I.D. No. 2: Drosophila *Nle* cDNA

15 aattcccaaaaaATGCAGGAGACGGACACGGAGCAAGAGGCCACGCCACATACG
ATACAGGCGCGCCTCGTTTACACGGGCGAGGAAGCCGGCCCGCCAATCGA
CCTGCCGGCAGGAATCACTACCCAGCAATTGGGACTGATTTGCAACGCGC
TGCTGAAAAACGAGGAAGCCACTCCATATTTGTTTTTCGTGGGCGAGGAT
20 GAGATCAAGAAGAGCCTGGAGGACACGTTGGACTTGGCGTCAGTGGACA
CCGAAAACGTGATCGATATTGTGTATCAGCCACAGGCGGTTTTCAAAGTG
CGCCCAGTGACAAGATGCACGAGTTCCATGCCGGGACACGCCGAGGCTGT
GGTTTCGCTGAATTCAGCCCGGATGGTGCTCATCTCGCCAGTGGAAGTG
GCGACACCACAGTGCGATTGTGGGATCTTAACACAGAGACACCGCACTTC
25 ACCTGCACAGGTCATAAGCAGTGGGTTCTGTGCGTATCCTGGGCTCCGGA
TGGCAAACGGTTGGCCAGCGGTTGCAAAGCGGGCTCTATAATCATCTGGG
ACCCGGAGACGGGTCAGCAGAAGGGGCGACCCTTGAGTGGGCACAAGAA
ACACATCAACTGCCTCGCCTGGGAACCGTATCATCGCGATCCGGAGTGCA
GGAAACTTGCTTCCGCCAGTGGAGACGGGGACTGCCGGATTTGGGACGTA
30 AAATTGGGCCAGTGCCTTATGAACATTGCCGGACACACAAATGCTGTGAC
AGCAGTGAGATGGGGTGGAGCGGGCCTTATTTATACATCCTCCAAAGATC
GCACAGTGAAGATGTGGCGAGCAGCTGATGGAATCTTGTGCCGGACGTTC
TCTGGCCAAGCTCACTGGGTAAACAACATTGCGCTGAGCACCGATTACGT
CCTGCGCACTGGTCCATTCCATCCGGTGAAGGATCGCTCCAAGAGCCACC

TCAGTTTGAGCACTGAGGAATTGCAGGAATCTGCCTTGAAGCGCTACCAG
GCCGTGTGCCCTGACGAGGTGGAGTCGCTGGTTTCCTGTTTCGGATGACAA
CACCTCTATCTGTGGCGGAACAACCAGAACAAGTGCGTTGAGCGCATGA
CAGGGCACCAGAACGTGGTCAACGATGTGAAATATTCGCCGGATGTAAAG
5 CTAATTGCGTCTGCTTCATTTGACAAGTCAGTGCGTCTGTGGCGAGCCAGC
GATGGTCAGTACATGGCCACCTTCCGGGGTCATGTGCAGGCTGTTTACAC
GGTTGCCTGGTCCGCGGACTCCCGCTTGATTGTTTCCGGCAGCAAAGACTC
AACTCTAAAAGTATGGAGTGTGCAGACGAAGAAACTGGCACAGGAGCTG
CCTGGACATGCGGATGAGGTGTTTCGGAGTGGACTGGGCGCCCGATGGCTC
10 TAGAGTTGCCTCTGGTGGCAAGGACAAAGTTATAAAGCTATGGGCTTATT
AAcaaatcattaactgtacacggttaagaaaatacttaggaataaagtaaacgtcctgagtaaaaaaaaaaaaaaaaaa
aaaaaaaaaaaaaaaaaaaaaa

REGULATOR OF NOTCH SIGNALING ACTIVITY

The present invention relates to a novel regulator protein and to nucleic acid that encodes this protein. The invention also relates to methods of diagnosis and therapy of disease using this protein and nucleic acids encoding therefor.

- 5 The Notch signalling pathway comprises an intracellular signalling mechanism that is essential for proper embryonic development. This pathway was first identified and studied in *Drosophila*, although comparative analyses have since demonstrated a remarkable degree of functional conservation between many of these developmentally important genes in *Drosophila* and their vertebrate counterparts. The Notch locus has
10 been shown to comprise an evolutionarily conserved cell interaction mechanism that plays a fundamental role in controlling the progression of immature cells to a more differentiated state.

- The *Notch* genes encode transmembrane receptors that help to determine cell fate during development. Notch acts as a receptor in a signalling pathway that when activated, may
15 block or delay the progression of immature cells toward a more committed state. Inappropriate activation or inactivation of Notch can lead cells to adopt an incorrect fate.

- The mechanism of signalling downstream of Notch receptors remains uncertain. However, it is thought that a proteolytic cleavage releases the cytoplasmic portion of
20 Notch which then translocates to the nucleus and participates in the specific activation of genes whose products inhibit differentiation.

- Mutations in *Notch* genes are linked to various diseases including some cancers (Zagouras *et al*, 1995; Capobianco *et al*, 1997; Gallahan and Callahan, 1997; Gridley, 1997). Indeed, two members of the mammalian Notch gene family were initially
25 identified as oncogenes and at least three distinct members of the Notch gene family (Notch 1, Notch 2 and Notch 4) can contribute to neoplasia in mammals. It seems that truncation of human Notch 1 plays a causal role in the formation of T cell neoplasms. Furthermore, an association has been shown between mutations in the Notch3 gene and the CADASIL phenotype (cerebral autosomal dominant arteriopathy with subcortical

infarcts and leukoencephalopathy). Notch function may also be linked to neurodegenerative disease by virtue of its connection to Presenilin (Levitan and Greenwald, 1995; de Strooper *et al*, 1998). This assertion is based on the study of genetic interactions in *C. elegans* in which a putative functional link between Notch and presenilin gene products has been noted. Presenilins are linked to processing of amyloid precursors, suggesting a potential role in the genesis of Alzheimer's disease.

However, the demonstration of a link between a particular disease and a specified genotype does not directly facilitate the development of a therapeutic strategy to treat or prevent the disease. This is particularly true for those linkages set out above, since although ligands of Notch have been identified, the signalling cascade downstream of the receptors remains incompletely understood.

It is likely that genes encoding proteins that regulate Notch signalling or that participate in Notch signalling may also be linked to various disease states. Identification of genes that modify Notch activity may thus provide the basis for a nucleic acid or protein diagnostic or therapeutic tool.

There is thus a need for the identification of molecules that affect the levels of activity of Notch and that might act as suitable targets for therapeutic agents for treatment of diseases linked to mutations in the Notch locus. Such proteins would also be useful in identifying drugs that modify Notch activity.

Summary of the invention

According to a first aspect of the present invention there is provided a protein comprising an amino acid sequence as identified in SEQ. ID. No. 1, or a functional equivalent thereof.

This novel protein, termed Notchless, was identified in a screen for dominant modifiers of a Notch mutant phenotype in the *Drosophila* wing. The mutant dominantly suppresses the wing notching phenotype of *notchoid* mutations and the Notchless protein is shown herein to bind to the cytoplasmic domain of Notch. Notchless modifies Notch signalling activity in a variety of Notch-dependent signalling processes in both *Drosophila* and *Xenopus* embryos.

By the term "functional equivalent" is meant any compound that possesses the same conformation as a domain of the Notchless protein that is responsible for its physiological function. Accordingly, this term is meant to include any macromolecule or molecular entity that mimics the conformation of the Notchless protein or that possesses an equivalent complementarity of shape to that possessed by the binding sites of the Notchless protein whose sequence is identified in SEQ ID 1.

Included in the invention as functional equivalents are invertebrate and vertebrate homologues of the *Drosophila* Notchless protein, with the proviso that the protein is not the *Xenopus* protein, the sequence of which is available on the accession number AF069737.

Also available on the genome database is a mouse EST (AA396500), a human EST (AA341327) and an *S. cerevisiae* DNA sequence (1351791). The *C. elegans* sequence was compiled by the inventors from multiple clones (C48486, D70156, C35601, M89091) and has a gap in the 6th WD40 repeat (this sequence was not identified as a gene by the *C. elegans* genome project). Until now, no function has been ascribed to any of these nucleic acid sequences in regulating Notch.

The term "functional equivalents" is also intended to include fragments or variants of the Notchless protein or closely related proteins exhibiting significant sequence homology. By "fragments" is meant any portion of the entire protein sequence that retains a physiological function of the wild type Notchless protein, such as for example, an ability to bind specifically to Notch. Accordingly, fragments containing single or multiple amino acid deletions from either terminus of the protein or from internal stretches of the primary amino acid sequence form one aspect of the present invention. "Variants" include mutants containing amino acid substitutions, insertions or deletions from the wild type Notchless sequence.

The Notchless protein is thought to function by binding to the cytoplasmic domain of Notch. It is thought (although the applicant does not wish to be limited by this theory) that Notchless also binds to protein factors other than Notch, since mutants that reduce or remove Notchless expression have been shown to increase Notch activity (see Examples).

The predicted Notchless protein has a novel highly conserved N-terminal domain followed by 9 WD40 repeats (Figure 6A). The WD40 repeat is found in a wide variety of proteins of diverse function and is thought to be a protein interaction domain (reviewed in Neer *et al.*, 1994). Typically, WD40 proteins contain 7 repeats. Structure analysis of β -transducin suggests that these form a propeller-like structure and that 7 repeats can pack to make a flat cylinder (Neer and Smith, 1996).

Notchless is unusual in that it appears to contain 9 WD40 repeats. Repeats 5 and 6, though recognisable as WD motifs, appear quite divergent in that they lack particular signature residues of the WD40 repeat (not shown).

- 10 Blast searches using the amino-terminal sequence (before the first WD repeat) identified closely related sequences identified in yeast, *C. elegans*, man and mouse. In all cases the N-terminal domain is followed by WD repeats. In the human and mouse, only short EST fragments have been sequenced; until now, no function has been assigned to these sequences.
- 15 Degenerate PCR using primers directed against conserved sequences in the N-terminal domain of the mouse and human proteins was used to isolate a *Xenopus Nle* cDNA. The *Xenopus* protein also contains 9 WD repeats with strong similarity to the *Drosophila* and *C. elegans* proteins. We note that particular WD40 repeats are more similar between species than they are to other WD40 repeats in the protein of the same species.
- 20 Together this suggests that these proteins represent true orthologues. Database searches suggest that there may only be one member of this gene family in *C. elegans*, mouse and the human.

Sequence comparison indicates that the degree of conservation in the N-terminal domain is quite high among the different family members (Figure 6B). In the 80 amino acid region corresponding to residues 27-106 of Notchless, sequence identity ranges from 33% between *Drosophila* and *S. cerevisiae* to 61% between *Drosophila* and *Xenopus* proteins. Particular residues are identical in all species examined, suggesting that they may be important for domain structure. This domain has been termed the Nle domain.

Orthologues of the Notchless protein are thus thought to retain their function across different species and to effectively regulate the activity of a protein member of the Notch gene family from a different species. By gene family is meant a group of genes whose products share a common function and that exhibit common sequence homology.

- 5 By common sequence homology is meant that the gene sequences are related by divergence from a common ancestor. The protein products of the genes may share common motifs in their sequence.

- According to a further aspect of the present invention there is provided a Notchless protein or functional equivalent thereof modified at one or more positions in the sequence of the
10 protein by the substitution, insertion or deletion of one or more amino acids from the wild type sequence shown in SEQ ID No. 1. Furthermore, such proteins may contain single or multiple amino acid deletions from either terminus of the protein or from internal stretches of the primary amino acid sequence.

- The Notchless protein or functional equivalent thereof may be prepared by any suitable
15 means as will be clear to the man of skill in the art. Preferably, the protein is generated by recombinant DNA technology by expression of the encoding DNA in an expression vector in a host cell. Such expression methods are well known to those of skill in the art and many are described in detail in *DNA cloning: a practical approach, Volume II: Expression systems*, edited by D.M. Glover (IRL Press, 1995) or in *DNA cloning: a practical approach, Volume IV: Mammalian systems*, edited by D.M. Glover (IRL Press,
20 1995). Protein compounds may also be prepared using the known techniques of genetic engineering such as site-directed or random mutagenesis as described, for example, in *Molecular Cloning: a Laboratory Manual: 2nd edition*, (Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press) or in *Protein Engineering: A practical approach* (edited
25 by A.R. Rees *et al.*, IRL Press 1993).

- For many applications, a protein according to the present invention may be fused to an effector or reporter molecule such as a label, toxin or bioactive molecule. Such molecules may comprise an additional protein or polypeptide fused to the Notchless protein, or functional equivalent, at its amino- or carboxy-terminus or added internally. The purpose
30 of the additional polypeptide may be to aid detection, expression, separation or purification of the protein or may be to lend additional properties to the protein as desired.

In particular, it is envisaged that fusion proteins comprising Notchless, or a functional equivalent thereof may be used as a "platform" to deliver biologically active protein domains to Notch. For example, an enzyme could be fused to Notchless or to a functional equivalent, that could be targeted to Notch in this fashion. Such a fusion partner would preferably be regulated in some fashion so that it would only be activated when the Notch and Notchless proteins physically interact. This may be through some conformational change, so that only when the Notch binding domain of Notchless interacts with the Notch protein is the fusion partner activated. Another mechanism may be through the inclusion in the fusion protein of a regulatable domain such as a hormone regulation domain, so that the activity of the fusion partner may be limited until the relevant drug is provided.

A particular enzyme of choice as a fusion partner might be a protease. Such an enzyme would act to "clip" the activated form of Notch that is expressed in some cancers, so inactivating it. As will be clear to those of skill in the art, any effector protein (or effector domain) could be used that is capable of modifying Notch activity *in vivo*. Post-translational modifications, such as phosphorylation (where kinase or phosphatase is the effector enzyme), or ubiquitination would also have the desired effect.

Other suitable candidates for fusion will be reporter molecules such as luciferase, green fluorescent protein, or horse radish peroxidase. Labels of choice may be radiolabels or molecules that are detectable spectroscopically, for example fluorescent or phosphorescent chemical groups. Linker molecules such as streptavidin or biotin may also be used. Additionally, other peptides or polypeptides may be fused to a Notchless protein. Suitable peptides may be, for example, β -galactosidase, glutathione-S-transferase, luciferase, polyhistidine tags, secretion signal peptides, the Fc region of an antibody, the FLAG peptide, cellulose binding domains, calmodulin and the maltose binding protein. Antibodies or peptides used to target the Notchless protein more efficiently towards a site of action (for example antibodies against membrane proteins of mast cells) may also be fused to the Notchless protein.

These fusion molecules may be fused chemically, using methods such as chemical cross-linking. Suitable methods will be well known to those of skill in the art and may comprise for example, cross-linking of the thiol groups of cysteine residues or cross-linking using

formaldehydes. Chemical cross-linking will in most instances be used to fuse non-protein compounds, such as cyclic peptides and labels.

When it is desired to fuse two protein molecules, the method of choice will often be to fuse the molecules genetically. In order to generate a recombinant fusion protein, the genes or
5 gene portions that encode the proteins or protein fragments of interest are engineered so as to form one contiguous gene arranged so that the codons of the two gene sequences are transcribed in frame.

According to a further aspect of the invention there is provided a process for the identification of an agent capable of modifying the levels of expression or activity of a
10 Notch protein comprising screening a Notchless mutant in a sensitised Notch genetic background with a drug and selecting for an altered phenotype.

Preferably, the Notchless mutant is an insect larva. For example, compound screening may be performed by feeding larvae of a suitable genetic background on food containing the compound of interest. Modified function is then assessed in the adult fly.
15 This allows the identification of compounds suitable for oral delivery that would either enhance or suppress the severity of the mutant phenotype through either increasing or decreasing the activity of Nle with respect to Notch.

One advantage of such a screen is that it makes possible the selection of drugs that modify Notch activity. Furthermore, these drugs will be suitable for oral delivery.
20 According to a further aspect of the present invention there is provided a drug identified by such a screen.

According to a further aspect of the present invention there is provided a nucleic acid sequence comprising:

- a) the sequence of SEQ ID No 2, or
- 25 b) a sequence that encodes on expression the amino acid sequence encoded by the sequence of part a), or
- c) a fragment of the DNA sequence of part a) or part b) that comprises a nucleotide sequence that encodes the binding domain of Notch.

The preferred nucleic acid molecule, according to the invention, comprises a nucleotide fragment identical to or complementary to any portion of the nucleotide sequence shown in SEQ ID No 2, that encodes a Notchless protein. It will be appreciated that individual or multiple nucleotide insertions, deletions and substitutions may be made
5 without departing from this aspect of the invention.

The nucleic acid molecule may comprise DNA, cDNA or RNA. Preferably, the nucleic acid molecule comprises DNA.

According to a further aspect of the invention there is provided a probe capable of screening for Notchless and prepared from the DNA sequence of SEQ ID No 2. The
10 probe preferably comprises at least 15 oligonucleotides, more preferably between 15 and 300 oligonucleotides, most preferably between 15 and 50 oligonucleotides.

The invention also includes cloning and expression vectors containing the DNA sequences of the invention. Such expression vectors will incorporate the appropriate transcriptional and translational control sequences, for example enhancer elements,
15 promoter-operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in frame with the nucleic acid molecules of the invention.

Additionally, in the absence of a naturally-effective signal peptide in the protein sequence, it may be convenient to cause the recombinant protein to be secreted from
20 certain hosts. Accordingly, further components of such vectors may include nucleic acid sequences encoding secretion signalling and processing sequences.

Vectors according to the invention include plasmids and viruses (including both bacteriophage and eukaryotic viruses). Many such vectors and expression systems are well known and documented in the art. Particularly suitable viral vectors include
25 baculovirus-, adenovirus- and vaccinia virus-based vectors.

The expression of heterologous polypeptides and polypeptide fragments in prokaryotic cells such as *E. coli* is well established in the art; see for example *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press or *DNA cloning: a practical approach, Volume II: Expression*

systems, edited by D.M. Glover (IRL Press, 1995). Expression in eukaryotic cells in culture is also an option available to those skilled in the art for the production of heterologous proteins; see for example O'Reilly *et al.*, (1994) *Baculovirus expression vectors - a laboratory manual* (Oxford University Press) or *DNA cloning: a practical approach, Volume IV: Mammalian systems*, edited by D.M. Glover (IRL Press, 1995).

Suitable vectors can be chosen or constructed for expression of Notchless proteins, containing the appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. bacteriophage, or phagemid, as appropriate. For further details see *Molecular Cloning: a Laboratory Manual*. Many known techniques and protocols for manipulation of nucleic acid, for example, in the preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel *et al.* eds., (John Wiley & Sons, 1992) or *Protein Engineering: A practical approach* (edited by A.R. Rees *et al.*, IRL Press 1993). For example, in eukaryotic cells, the vectors of choice are virus-based.

A further aspect of the present invention provides a host cell containing a nucleic acid encoding a Notchless protein or functional equivalent. A still further aspect provides a method comprising introducing such nucleic acid into a host cell or organism. Introduction of nucleic acid may employ any available technique. In eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection or transduction using retrovirus or other viruses, such as vaccinia or, for insect cells, baculovirus. In bacterial cells, suitable techniques may include calcium chloride transformation, electroporation or transfection using bacteriophage.

Introduction of the nucleic acid may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for allowing expression of the gene.

In one embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

Transgenic animals transformed so as to express or overexpress in the germ line one or more Notchless proteins or functional equivalents as described herein form a still further aspect of the invention, along with methods for their production. Many techniques now exist to introduce transgenes into the embryo or germ line of an organism, such as for example, illustrated in Watson *et al.*, (1994) *Recombinant DNA* (2nd edition), Scientific American Books.

10 According to a further aspect of the present invention there is provided a method of gene therapy of a pathological condition caused by a gene mutation in a patient comprising administering to a patient a nucleic acid encoding a Notchless protein or functional equivalent, in a therapeutically-effective amount.

Suitable diseases include various cancers such as human acute lymphoblastic leukaemia 15 (Capobianco *et al.*, 1997), cervical squamous carcinoma and adenocarcinoma (Zygouros *et al.*, 1995), and mammary tumours (Gallahan & Callahan, 1997; Gridley, 1997) and certain neurodegenerative diseases, for example familial Alzheimer's disease.

The nucleic acid may be introduced into a patient by any suitable means, as will be clear to those of skill in the art. Effective methods of introduction include the use of 20 adenovirus, adeno-associated virus, herpes virus, alpha virus, pox virus and other virus vectors that serve as delivery vehicles for expression of the gene. See generally, Jolly (1994) *Cancer Gene Therapy* 1:51-64; Kimura (1994) *Human Gene Therapy* 5:845-852; Connelly (1995) *Human Gene Therapy* 6:185-193; and Kaplitt (1994) *Nature Genetics* 6:148-153. Retroviral vectors may also be used (see *Tumor Viruses*, 25 Second Edition, Cold Spring Harbor Laboratory, 1985.) Preferred retroviruses for the construction of retroviral gene therapy vectors include Avian Leukosis Virus, Bovine Leukaemia, Virus, Murine Leukaemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis Virus and Rous Sarcoma Virus.

The term "therapeutically effective amount" as used herein refers to an amount of a 30 therapeutic agent to treat, ameliorate, or prevent the disease or condition, or to exhibit a

detectable therapeutic or preventative effect. The precise effective amount for a subject for a given situation can be determined by routine experimentation and is within the judgement of the clinician. An effective dose will typically be from about 0.01 mg/ kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of nucleic acid construct.

- 5 Non-viral strategies for gene therapy also exist that utilise agents capable of condensing nucleic acid molecules, delivering these molecules to cells and protecting them from degradation inside the cell. Vehicles for delivery of gene therapy constructs may be administered either locally or systemically.

Such strategies include, for example, nucleic acid expression vectors, polycationic
10 condensed DNA linked or unlinked to killed adenovirus alone (see Curiel (1992) *Hum Gene Ther* 3:147-154) and ligand linked DNA (see Wu (1989) *J Biol Chem* 264:16985-16987). Naked DNA may also be employed, optionally using biodegradable latex beads to increase uptake. Other methods will be known to those of skill in the art.

Liposomes can act as gene delivery vehicles encapsulating nucleic acid comprising a
15 gene cloned under the control of a variety of tissue-specific or ubiquitously-active promoters. Mechanical delivery systems such as the approach described in Woffendin *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91(24):11581-11585 may also be used.

Direct delivery of gene therapy compositions will generally be accomplished, in either a single dose or multiple dose regime, by injection, either subcutaneously,
20 intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions may also be administered directly into a tumour or lesion. Other modes of administration include oral and pulmonary administration, using suppositories, and transdermal applications, needles, and gene guns or hyposprays.

According to a further aspect of the invention there is provided a pharmaceutical
25 composition comprising a Notchless protein or functional equivalent according to the first aspect of the invention, in conjunction with a pharmaceutically-acceptable excipient. Suitable excipients will be well known to those of skill in the art and may, for example, comprise a phosphate-buffered saline (0.01M phosphate salts, 0.138M NaCl, 0.0027M KCl, pH7.4), a liquid such as water, saline, glycerol and ethanol, optionally also
30 containing mineral acid salts such as hydrochlorides, hydrobromides, phosphates,

5 sulphates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Auxiliary substances such as wetting or emulsifying agents, pH buffering substances, may also be present. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

10 A carrier may also be used that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Pharmaceutical compositions may also contain additional preservatives to ensure a long shelf life in storage.

15 According to a yet further aspect, the present invention provides a method of treatment of cancer or of a neurodegenerative disease in a patient comprising administering to a patient a Notchless protein or functional equivalent in a therapeutically-effective amount.

According to a yet further aspect, the present invention provides for the use of a Notchless protein or functional equivalent, a nucleic acid encoding a Notchless protein or functional equivalent or of a pharmaceutical composition containing a Notchless protein or functional equivalent in therapy.

20 According to a still further aspect of the invention there is provided the use of a Notchless protein or functional equivalent according to the invention in conjunction with a pharmaceutically-acceptable carrier in the manufacture of a medicament for the treatment or prevention of cancer or of a neurodegenerative disease in a human or an animal.

25 The invention also comprises the use of components according to the invention in a diagnostic kit for the detection of mutations present in the genome of a patient. Preferably, such a component will comprise a Notchless protein or functional equivalent, a nucleic acid encoding a Notchless protein or functional equivalent or an antibody that specifically recognises a Notchless protein or functional equivalent. Other desirable
30 components for inclusion in such a kit will be clear to those of skill in the art.

All documents mentioned in the text are incorporated herein by reference.

Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to the Notchless gene isolated from *Drosophila*. It will be appreciated that modification of detail may be made without
5 departing from the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Comparison of sequences of Notchless proteins from *Drosophila*, yeast, *C. elegans*, mouse, human and *X. laevis*.

Figure 2: Genetic interactions between *Notchless* and *notchoid*.

- 10 (A) Cuticle preparation of a wild-type wing.
- (B) Wingless protein expression in a wild-type wing imaginal disc visualized by antibody staining. The arrow indicates the stripe of Wingless at the DV boundary.
- (C) Cuticle preparation of a *nd¹* wing (genotype *nd¹/Y*; note that *Notch* is on the X-chromosome so males carry only one copy of the gene).
- 15 (D) *nd¹/Y; Nle^{k13714}/+* wing. The notching of the wing is completely suppressed.
- (E) *nd¹/Y; Su(H)^{AR9}/+*. Removing one copy of the *Su(H)* gene enhances the severity of the notching of the wing. Wingless expression in a disc of the same genotype is shown at right.
- (F) *nd¹/Y; Su(H)^{AR9}/Nle^{k13714}* wing. Removing one copy of *Nle* suppresses the
20 notching of the *nd¹/Y; Su(H)/+* wing and enhances the loss of veins. Wingless expression is restored to normal.
- (G) *nd^{fa}/Y* wing.
- (H) *nd^{fa}/Y; Nle^{k13714}/+* wing. The notching of the wing margin is completely suppressed. Veins are normal in this genotype.

Figure 3: Cloning the *Notchless* gene.

- (A) schematic representation of the *Nle* locus.
- (B) *Notchless* phenotype (suppressed *nd^l* phenotype) produced when one copy of *Nle* is mutated in a *nd^l* fly.
- 5 (C) Wing from a fly of the genotype as in panel B which also carried a UAS-*Nle* transgene on the second chromosome. Placing the 1.5 Kb transcript under C765-GAL4 regulation restores the *nd^l* phenotype (arrow).

Figure 4: *Notchless* enhances *Abruptex* mutant phenotypes

- (A) Wild-type wing, thorax and head cuticles. Veins 1-5 are numbered. The red arrows
10 in the central panel indicate two of the large bristles on the thorax. The blue shading in the right panel indicates the cluster of three orbital bristles above the eye.
- (B) *Abruptex²⁸* mutant wing, thorax and head cuticles.
- (C) *Abruptex²⁸ Nle^{Δ8/+}* mutant wing thorax and head cuticles.

Figure 5: Genetic interactions between *deltex* and *Notchless*

- 15 (A) *deltex^l* mutant wing.
- (B) *deltex^l Nle^{Δ8/+}* mutant wing.
- (C) Heat-shock Deltex overexpression under mild conditions produces no phenotype in an otherwise wild-type wing.
- (D) Comparable heat-shock Deltex treatment causes loss of veins in a *Nle^{Δ8/+}* wing
20 (arrow).

Figure 6: Molecular features of Notchless protein

- (A) Schematic representation of Notchless protein and its orthologues. The conserved Nle domain is indicated in dark gray. WD40 repeats are numbered 1-9 (white numbers).

Percent identity to the *Drosophila* protein are indicated for the Nle domain and for individual WD40 repeats.

- (B) Comparison of Nle domains. Sequence identity is highlighted in black, similarity in grey. Similarities are not highlighted if shared by fewer than four proteins. Dashes indicate gaps introduced to accommodate extra residues in the yeast protein. +15 aa indicates a larger insertion.

Figure 7: Expression of *Xenopus Notchless* during embryonic development and phenotypic effects of Notchless overexpression on formation of primary neurons.

- (A) Temporal expression of *XNle*.
 10 (B) Spatial expression of *XNle*.
 (C) Phenotypic consequence of overexpression of *XNle*, *DNle* and an activated form of *Xenopus NotchI* (XN-ICD) on primary neurogenesis.

Figure 8: Notchless binds Notch *in vitro*

- (A) Binding of Nle, Numb-N and Numb-C to GST-Notch-ICD and GST control
 15 proteins.
 (B) Immunoprecipitation of Notch and Nle expressed in S2 cells.

EXAMPLES

Materials and methods

Drosophila strains

- 20 l(2)k13714 is from the BDGP P-element lethal collection. P-element excisions were generated by providing a chromosomal source of transposase activity. 105 w⁺ excision lines were isolated. One of these was not able to suppress the *nd¹* phenotype and was therefore reverted to wild type. Others were analysed for imprecise excision of the P-element by Southern blots. *Su(H)^{SF8}* and *Su(H)^{AR9}* are described in Schweisguth and
 25 Posakony, 1992. *Ax²⁸* is described by de Celis and Garcia-Bellido, 1994. *deltex¹* and pCaSpeR hs-dx are described by Diederich, *et al.*, 1994; Matsuno *et al.*, 1995. *nd¹*, *nd²*,

nd fa and *Dp(1;2)51b* are described in Lindsley and Zimm (1992) "The genome of *Drosophila melanogaster*", Academic Press, San Diego. *w¹¹¹⁸* was used as wild-type control for cuticle preparations. For heat shock experiments, *pCaSpeR hs-dx/+* and *pCaSpeR hs-dx/Nle* pupae were heat shocked twice for 1 hour at 37°C between 0 and 24 hours after pupariation.

Antibodies

Mouse monoclonal anti-Wg is described in Brook and Cohen, 1996. Mouse monoclonal anti-Notch C17.9C6 is described in Fehon *et al.*, 1990. Mouse (12CA5) anti-HA and rabbit (HA-11) anti-HA were obtained from BabCo.

Constructs for rescue and expression

A 15Kb *SalI* genomic fragment of phage Y2-6 was inserted into the *XhoI* site of the transformation vector *pCaSpeR4*. *UAS-Nle* was prepared by cloning the 1.5Kb *Nle* cDNA as a *NotI-XhoI* fragment into *pUAST* (Brand and Perrimon, 1993). An HA-tagged version of *Nle* was generated by introducing three copies of the HA epitope (YPYDVPDYA) immediately downstream of the first Methionine residue. The *BamHI-AscI* fragment of *pKS-Nle* was replaced by a corresponding PCR fragment amplified using the following primers:

5' CGGATCCAAA AAATGTATCC CTATGACGTC CCCGATTATG
CCTACCCTTA CGATGTACCT GACTACGCGT ATCCGTACGA CGTTCCGGAC
20 TATGCTCAGG AGACGGACA CGGAGCAAGA GGCCACGCCA CATACGATAC
AGGCGCGCCA A 3' and

5' TAAACGAGGC GCGCCTATCG TAT 3'.

pMT-HA-Nle was generated by cloning *HA-Nle* as a *BamHI-SalI* fragment into the inducible expression vector *pRmHa-3*. *pRmHa-3-Notch* is described in Fehon *et al.*, 1990.

GST-fusion protein binding assay

GST-NICD was expressed in bacteria and purified as described in Guo *et al.*, 1996. ³⁵S-labelled Numb-N (aa 1-224), Numb-C (aa 224-547) and full length Nle were synthesised by in vitro transcription/translation using the TNT system (Promega). Binding reactions were carried out with 10 µl of labeled protein and 5 µl of GST or GST-NICD coupled beads in 400 µl of PBS 0.1% NP-40 for 1 hour at room temperature. The beads were washed 6 times in PBS, proteins eluted in SDS-gel sample buffer, separated on 10% SDS-polyacrylamide gels and visualised by autoradiography.

Immunoprecipitation

Schneider S2 cells were grown at 25°C in Schneider's medium (Gibco-BRL) with 1% fetal calf serum and 1% Gentamicin. Cells were harvested and transferred into 6-well 30 mm diameter tissue culture plates at 75% confluence. Each well was then rinsed 3 times with Schneider medium without serum and incubated with 10 µg of DNA in 500 µl of Schneider medium and 50 µl of Lipofectin (Gibco-BRL) for 6 hours. Cells were incubated overnight in medium without Lipofectin. Expression was induced by adding CuSO₄ to 0.7 mM and incubating for 12 hours. Cells were harvested and lysed by sonication in PBS, 50 mM NaCl, 5 mM EDTA, 5 mM DTT, 1% Triton X-100 containing protease inhibitors (1 mM PMSF, 5 µg/ml aprotinin and leupeptin). Cells debris was removed by 10,000 xG centrifugation. 500 µl of extract (corresponding to 1x10⁶ cells) was incubated with 3 µl of Rabbit anti-HA antibody for 1 hour at 4°C followed by 1 hour at 4°C with 20 µl of a 50% slurry of Protein A-Sepharose beads (Pharmacia). The beads were washed 4 times with lysis buffer, proteins eluted in SDS-gel sample buffer and run on a 6% SDS-polyacrylamide gel. The gel was electrophoretically transferred to Immobilon-P membrane (Millipore), blocked for 1 hour at room temperature in 5% dry milk in TTBS (10 mM Tris pH 8.0, 150 mM NaCl, 0.2% Tween-20) and incubated overnight at 4°C with mouse-anti Notch (9C6; used at 1:2000) or mouse anti-HA (1:1000). The membrane was washed 3 times 5 min in TTBS and incubated for 1 hour with peroxidase-conjugated goat-anti-mouse IgG (Jackson labs) diluted 1:5000 in TTBS. The blot was washed 3 times for 5 min in TTBS and developed using ECL reagents (Amersham).

Example 1: Genetic characterisation of a novel modifier of *Notch* activity

*notchoid*¹ (*nd*¹) is a viable mutant allele of *Notch* that causes scalloping of the wing (Figure 2C). The severity of the *nd*¹ phenotype is sensitive to the level of activity at other loci encoding components of both the Notch and Wingless signalling pathways. Thus *nd*¹ provides a sensitised genetic background in which to screen for modifiers of Notch signalling activity.

The BDGP collection of P-element induced lethal mutations (Spradling *et al.*, 1995) was screened for dominant modifiers of the *nd*¹ phenotype. Several P-element induced mutants were found to enhance the severity of *nd*¹ (not shown). One P-element induced mutant, l(2)k13714, was found that suppresses the scalloping of *nd*¹ wings (Figure 2C, D). On the basis of its ability to dominantly suppress scalloping of the wing, the gene identified by the l(2)k13714 P-element was called *Notchless* (*Nle*).

To verify that the gene mutated by the P-element is responsible for the mutant phenotype, strains were generated in which the original P-element had been removed by transposase-mediated excision. These chromosomes differ from the original l(2)k13714 chromosome only by the lack of the P-element and fail to suppress the *nd*¹ phenotype (data not shown). Although l(2)k13714 comes from a collection of P-elements that are supposed to be lethal mutations, it was noted that homozygous mutant individuals are recovered in this stock. They are morphologically normal, though males are sterile (not shown).

The scalloping of *nd*¹ mutant wings is thought to be caused by reduced Wingless activity because overexpression of Wingless can suppress the phenotype (Couso and Martinez Arias, 1994) and because further reducing *wingless* activity enhances the *nd*¹ phenotype (Hing *et al.*, 1994). Removing one copy of the *Su(H)* gene enhances the severity of the *nd*¹ phenotype and causes an obvious reduction of Wingless expression at the DV boundary (relative to the level in wild-type, compare Figure 2B and E; *nd*¹ *Su(H)/+*). Wingless is restored to wild-type levels and the loss of wing tissue is completely suppressed when the *Notchless* mutant is introduced in this background (Figure 2F; *nd*¹ *Su(H)/Nle*). *Notchless* also suppresses the phenotypes of *nd*^{fa} (Figure

2G, H) and *nd*² (data not shown), indicating that the genetic interaction is not specific to one particular allele of *Notch*.

The scalloping phenotype of *nd* alleles is thought to be due to reduced Notch function. Notch signalling through Su(H) is required to induce Wingless at the wing margin
5 (Diaz-Benjumea and Cohen, 1995). Reducing *Su(H)* gene dosage enhances the *nd*¹ phenotype. Introducing one copy of the *Notchless* mutant was found to restore Wingless expression in the *nd*¹ *Su(H)*/+ background. This suggested that reducing Notchless activity increases Notch activity at the DV boundary of the wing disc.

Example 2: Cloning the Notchless gene

10 The P element insertion in l(2)k13714 was mapped to cytological position 21C7-8 by the BDGP (Flybase), between the breakpoints of two large deletions Df(2L)al and Df(2L)ast¹ (Figure 3A). Neither of these deletions acts as a dominant suppressor of *nd*¹ (data not shown), suggesting that the *Notchless* gene lies in the interval between them.

DNA flanking the P element was cloned by plasmid rescue using *EcoRI* digested
15 genomic DNA. A 2.5Kb *EcoRI-HindIII* fragment (devoid of P-element sequences) was used to screen a chromosomal walk kindly provided by Markus Noll (Institut für Molekularbiologie II der Universität Zürich, Switzerland). The rescue fragment hybridised to a 3.3Kb *EcoRI* fragment. Sequencing of the 3.3Kb DNA fragment revealed the presence of open reading frames on both sides of the P-element insert but
20 in opposite orientation. Genomic rescue suggested that the gene was encoded by the 1.5Kb transcript (to the right of the insert in Figure 3). A 1.1Kb *EcoRI-ClaI* fragment to the right of the insertion site containing part of the predicted transcription unit was used to screen a λ gt10 eye disc cDNA library (kindly provided by G. Rubin; University of California, Berkeley CA). 6 cDNA clones were isolated. One encodes a putative full-
25 length *Nle* cDNA of 1.5Kb. The rescued DNA hybridised to a 3.3Kb *EcoRI* fragment of λ phage Y2-6. Sequence of the genomic flank identified transcription units on both sides of the P-element insertion (Figure 3A).

The 1.5Kb transcript was identified as the *Notchless* gene by two criteria: (i) the 15Kb *Sall* fragment of phage Y2-6 was able to restore *Notchless* activity when introduced into

- a *nd^l Nle/+* mutant background (data not shown). The transgene contains all of the 1.5Kb transcription unit but only part of the other transcription unit. (ii) Expression of the 1.5Kb cDNA under GAL4 control restores full *Nle* activity. *nd^l; Nle/+* mutant flies carrying a GAL4 driver show the suppressed *nd^l* phenotype (Figure 3B). The wing notching phenotype was restored when the 1.5Kb transcript is expressed in the wing disc under GAL4 control in the *nd^l; Nle/+* mutant (compare Figure 3B and C). Thus increasing the amount of *Notchless* product using GAL4 counteracts the effects of the *Nle* mutant and alleviates the suppression of the *nd^l* mutant phenotype. This indicates that the *Nle* mutant phenotype is due to reduced gene activity.
- 10 The P-element insertion that causes the mutation is located 310 bp 5' to the start of the *Nle* open reading frame. It is therefore likely that the P-element mutant reduces the level of *Nle* expression. To obtain a deletion that removes the *Nle* locus, mutants were generated by mobilisation of the P-element. An excision mutant named *Nle Δ 8* deletes sequences on both sides of the insertion (Figure 3A).
- 15 To ask whether the *Nle Δ 8* deletion allele would produce a stronger increase in Notch activity than the l(2)k13714 P-element insertion mutant, suppression of the *nd^l* phenotype were first examined. We observed no difference in the extent of suppression of *nd^l* (data not shown). The *Nle Δ 8* deletion is embryonic lethal when homozygous, but deletes at least one additional transcription unit. Bearing in mind that any phenotypes produced by the deletion could be attributed to its being mutant for more than one gene,
- 20 homozygous *Nle Δ 8* embryos and clones of *Nle Δ 8* mutant cells were examined for neurogenic phenotypes. No difference was detected between mutant and wild-type embryos in the developing PNS and CNS, visualised by 22C10 antibody (data not shown). Likewise, we did not observe any bristle pattern abnormality in the notum or
- 25 wing of homozygous *Nle Δ 8* mutant clones (data not shown).

Flies heterozygous for the P-element insertion and the *Nle Δ 8* deletion are viable, morphologically normal and male sterile, like the homozygous P-element mutant. Together these observations suggest that the original P-element mutant may be a null

allele of *Nle*. The lethality caused by the *Nle* Δ^8 deletion is likely to be due to another gene.

Example 3: *Notchless* enhances the effects of mutants that increase Notch activity

Certain *Abruptex* alleles of *Notch* have been classified as mutations that increase Notch activity. Their phenotypes are enhanced by increasing the level of wild-type *Notch* gene product and are suppressed by reducing it (de Celis and Garcia-Bellido, 1994; Brennan *et al.*, 1997). Like other gain-of-function *Abruptex* alleles *Ax*²⁸ flies show reduced numbers of some bristles on the head and thorax, as well as shortening of wing veins (Figure 4A, B). These phenotypes are made more severe by introducing an extra copy of the wild-type *Notch* gene (data not shown). They are also enhanced by removing one copy of the *Notchless* gene (Figure 4C). The shortening of the wing veins is more pronounced in *Ax*²⁸ *Nle*/+ flies (arrows). *Ax*²⁸ *Nle*/+ flies show increased loss of both small bristles in the thorax (note the large bare patch outlined in red, Figure 4C), and of large bristles in the head compared with *Ax*²⁸ flies. Blue shading on the head indicates the cluster of orbital bristles. There are three in wild-type flies, one or two in *Ax*²⁸ flies and none in *Ax*²⁸ *Nle*/+ flies. Thus removing one copy of *Nle* enhances the severity of the phenotypes caused by increased Notch activity in *Ax*²⁸ flies.

It was observed that wing veins are reduced in mutant combinations involving *nd*¹ and *Nle*/+ (Figure 2D, F). Similar results were obtained with *nd*² (data not shown). This phenotype is likely to reflect increased Notch activity. Matsuno *et al.*, (1995) have observed loss of wing veins in *nd*¹ heterozygous flies (which are themselves morphologically normal) when a low level of the activated form of Notch is expressed under heat-shock control. Together these observations suggest that the *nd*¹ mutation shows an abnormal increase in Notch activity in wing vein formation. By analogy to the effects of expressing the activated form of Notch (Matsuno *et al.*, 1995) it is probable that the effect of the *Nle* mutation is to further increase the aberrant Notch activity in the *nd*¹ mutation. These results appear to be at odds with the observation that the *nd*¹ mutation reduces Notch function at the wing margin (Figure 2). This suggests that the

nd¹ mutation behaves as a loss of function allele in one context and as a gain of function allele in another.

Example 4: *Notchless* opposes *deltex* function

Deltex is thought to function as a positive regulator of Notch activity (Diederich *et al.*, 1994; Matsuno *et al.*, 1995). *deltex* mutant flies show a phenotype resembling a reduction of Notch activity: nicking of the distal region of the wing blade and thickening of the wing veins (Figure 5A). Removing one copy of *Notchless* restores the *deltex* mutant wing to normal (Figure 5B). Thus the effects of reducing *deltex* activity can be compensated for by simultaneously reducing *Notchless* activity. Likewise, removing one copy of *Notchless* enhances the effects of overexpressing Deltex using a heat shock-*deltex* transgene (Matsuno *et al.*, 1995). Under conditions where Deltex overexpression produces no visible abnormality in an otherwise wild-type wing (Figure 5C), it causes loss of veins in a *Nle/+* background (arrow, Figure 5D). This resembles the effects of increasing Notch activity in *Abruptex* mutants.

These results suggest that Deltex and Notchless act in opposite directions as modifiers of Notch activity in wing development.

Example 5: *Notchless* expression in *Xenopus*

XNle was isolated by PCR using the degenerate primers, F 5'-CGC AGA ATT CCI TTY GAY GTI CCI GTI GAY AT-3' and R 5'-GGT GCT CGA GCY TGI GGY TGR TAI ATD ATR TC-3', designed against conserved peptides, PFDVPVDI and DIIYQPQ respectively, found in the Nle domain of the vertebrate proteins identified as expressed sequence tags.

Phage stock of a stage 30 library (Stratagene) was used as template to amplify a 200 bp fragment that spans the Nle domain. Five independent clones were sequenced and found to be identical. This fragment was used to screen the stage 30 library, which resulted in the isolation of 25 positive clones of which the longest of 2.2Kb was sequenced on both strands. Temporal expression was assayed by RT-PCR analysis as described by Bouwmeester *et al.*, 1996 using the following primer set that amplifies a *XNle* fragment

of 135 bp; F 5'-CAC CAG ATA AAC TGC AGT TAG-3', R 5'-CTG TTT CAA CTG ATT GCT TCT-3' (28 cycles).

Spatial expression was analysed by whole-mount in situ hybridisation essentially as described (Bouwmeester *et al.*, 1996), using antisense RNA synthesised from pBS-*XNle* linearised with *XhoI* and transcribed with T3 polymerase.

For injection purposes pCS2-*XNle* was constructed by subcloning of a 2.2Kb *EcoRI* fragment in the complementary site of pCS2+. Capped RNA was synthesised using pCS2-*XNle*, pCS2-*Drosophila Nle* (kindly provided by J. Wittbrodt; Max-Planck Institut für Biophysikalische Chemie, Göttingen, Germany) and pCS2-*NOTCH1-ICD* (kindly provided by C. Kintner; Salk Institute, San Diego CA) digested with Not-1 and transcribed with Sp6. Synthetic RNA (2.5 - 5 ng of *XNle* and *DNle* RNA, 100-200 pg *XN-ICD*) was injected into one blastomere of the 2-cell stage embryo. Embryos were harvested at early neurula stage (st. 13-15). β -galactosidase activity, a lineage marker for injections, was revealed using X-gal as substrate prior to whole mount in situ. Primary neurons were identified by β -tubulin staining. Antisense β -tubulin RNA was synthesised from pBS- β -tubulin digested with Not I and transcribed with T3 polymerase.

Figure 7 shows the temporal expression of *XNle*. Total RNA isolated from the indicated stages of development was analyzed by RT-PCR analysis for expression of *XNle* and *Histone H4* (loading control). E, egg; 4C, 4 cell stage; all other lanes are labelled with stage numbers according to Nieuwkoop and Faber (1956) Normal table of *Xenopus laevis* A. Systematical and chronologica survey of the development from the fertilised egg until the end of metamorphosis. North Holland publishing company, Amsterdam. (B) Spatial expression of *XNle*. Whole mount in situ hybridization was used to visualize expression of *XNle* at neural plate stage (st. 17), tailbud stage (st. 25) and tadpole stage (st. 35). Expression patterns are described in the text. symbols: NC, neural crest; pm, paraxial mesoderm; b, brain; e, eye; ba, branchial arches; s, somites; sp, segmental plate; vbi, ventral blood islands. (C) Phenotypic consequence of overexpression of *XNle*, *DNle* and an activated form of *Xenopus Notch1* (XN-ICD) on primary neurogenesis. LacZ RNA was co-injected to mark the injected side. Control embryo: l, i, m denote lateral, intermediate and medial rows of β -tubulin expressing primary

neurons. Note the reduction in the number of primary neurons on the injected side in embryos injected with *XNle*, *DNle* or *N-ICD*. Arrows indicate the absence of lateral and intermediate neurons in *XNle* and *DNle* injected embryos and all neurons in *XN-ICD*-injected embryo. In B and C anterior is to the left.

- 5 This figure shows that the *Xenopus Notchless* gene (*XNle*) is maternally transcribed and that expression remains relatively constant during the early stages of embryonic development without obvious signs of localisation. Elevated levels arise at the end of gastrulation and are maintained during neurulation and organogenesis (Figure 7A). Localised expression is observed in two lateral domains adjacent to the rostral neural
10 plate, which correspond to the premigratory neural crest cells, and in a region at the anterior end of the neural plate, which corresponds to placodal precursors (Figure 7B). There is also increased expression in the involuting paraxial mesoderm and in two patches lateral to the closing slit blastopore, through which future somitic cells involute.

- During subsequent stages expression is evident in the somites and unsegmented paraxial
15 mesoderm, the segmental plate. High levels are also seen in the head region; in the branchial arches, eyes and different regions of the developing brain (Figure 7B, st.25). Later on expression is in addition found in two patches on the ventral site of the embryo, the ventral blood islands which generate the hematopoietic precursors of the early embryo (Figure 7B, st.35).

- 20 The pattern of *XNle* expression resembles that of other components of the Notch pathway, including *Delta* and *Kuzbanian* (Chitnis *et al.*, 1995; Pan and Rubin, 1997). These expression domains correspond to regions where Notch signalling has been implicated in cell fate specification events (Chitnis *et al.*, 1995; Coffman *et al.*, 1993; Jen *et al.*, 1997).

25 **Example 6: Overexpressing Notchless increases Notch activity**

- Based on the finding that reducing *Nle* activity increases Notch activity in *Drosophila* (Figs 1-4), it was anticipated that overexpression of *Nle* would reduce Notch activity. To test this proposal the *Xenopus* neuronal specification assay was used (Chitnis *et al.*, 1995). Notch signalling is involved in controlling the choice between neural and
30 epidermal fate. Overexpression of activated forms of Notch reduces the number of cells

adopting neural fate in *Xenopus* (Chitnis *et al.*, 1995). Conversely, reduction of Notch activity would be expected to increase the number of cells adopting neural fate, as in *Notch* mutant embryos in *Drosophila* (Campos-Ortega and Jan, 1991). Surprisingly, it was observed that overexpression of XNle and of *Drosophila* Nle reduces the number of
 5 neurons, as in the activated Notch control (Figure 7C). Although high levels of *Nle* RNA were injected, no sign of other developmental defects was observed: gastrulation and subsequent morphogenesis proceeds normally.

This unexpected finding led us to test whether overexpression of Nle in *Drosophila* would have a comparable effect on neural fate specification. Expression of activated
 10 Notch reduces thoracic bristle formation (Struhl *et al.*, 1993; Rebay *et al.*, 1993). *UAS-Nle* was expressed in the notum under control of apterous-GAL4. The number of small bristles was found to be reduced in flies expressing *UAS-Nle* compared to *apterous-GAL4* alone (Table 1). Although the reduction is not large in magnitude, it is statistically significant ($p < 0.0001$). Thus overexpression of Nle increases Notch activity
 15 in both *Xenopus* and *Drosophila* neural fate specification.

Table 1: Suppression of neural fate by overexpression of Notchless

# microcheate/notum		
<u>genotype</u>	<u>mean \pm SE (n)</u>	<u>t-test</u>
20 ap-Gal4/+	214 \pm 2 (17)	
ap-Gal4/UAS-Nle	196 \pm 3 (17)	$p < 0.00001$

Wild-type flies have on average 260 small bristles per thorax (Brennan *et al.*, 1997). This number is reduced in Apterous-GAL4/+ flies. Expression of Nle further reduces
 25 the number of bristles.

Example 7: Notchless protein binds to the intracellular domain of Notch -

To ask whether Nle might regulate Notch through direct protein interaction we carried out GST pull down and immunoprecipitation assays. *In vitro* binding was tested using ³⁵S-Met-labeled test proteins and the intracellular domain of Notch expressed in bacteria as a GST-fusion protein (Guo *et al.*, 1996). Figure 8A shows binding of Nle, Numb-N and Numb-C to GST-Notch-ICD and GST control proteins. N-terminal and C-terminal fragments of Numb were used as controls for binding to the intracellular domain of Notch (Guo *et al.*, 1996). Input lanes show 1/10 of the input to the binding reaction. GST-N indicates GST-Notch-ICD beads; GST indicates GST-beads. Numb-N and Nle (arrow) bind to GST-N beads more strongly than to GST control beads. Coomassie blue staining of the gel (not shown) showed that there was significantly more protein bound to the GST control beads than to the GST-N beads.

To conclude, Figure 8A shows weak non-specific binding of GST control beads to all three proteins, but this is well below the level of specific binding observed with Numb-N and Notchless.

In vivo interaction between Notchless and Notch in *Drosophila* S2 cells was tested by immunoprecipitation. Expression of full-length Notch and HA-tagged Notchless proteins was monitored by immunoblotting of total cell extracts (see below). Extracts from induced and uninduced cells were immunoprecipitated using antibody to the HA-tag, and a blot of the gel was probed with a monoclonal antibody directed against the intracellular part of Notch and reprobed subsequently with anti-HA to visualise the immunoprecipitated HA-Notchless.

Figure 8B shows the results of the immunoprecipitation of Notch and Nle expressed in S2 cells. Upper panel: blot probed with mouse monoclonal anti-Notch (9C6). Lower panel, same blot probed subsequently with mouse anti-HA. Lanes 1-3: total cell lysates from S2 cells expressing (1) Notch (2) HA-Nle or (3) both proteins. Note that low levels of endogenous Notch are seen in the Nle-expressing cells (lane 2). Lanes 4-7: Immunoprecipitates from cells expressing (4)Notch, (5) HA- Notchless or (6, 7) both proteins. + indicates immunoprecipitated with Rabbit anti-HA and protein A beads. - indicates control precipitation with protein A beads alone. Immunoprecipitation of HA-Nle coprecipitates Notch in cells that overexpress both proteins (lane 7). Neither protein

is recovered in control precipitations lacking the anti-HA (lane 6) or in which HA-Nle was not expressed (lane 4). Immunoprecipitation in cells transfected with HA-Nle alone did not detectably coprecipitate Notch (lane 5), although endogenous Notch can be detected in cells not transfected with the inducible Notch expression construct (lane 2).

- 5 Recovery of HA-Nle was lower in the reaction in lane 5 than in lane 7.

Notch protein was thus found to immunoprecipitate with HA-Notchless from cells expressing both proteins (Figure 8B lane 7). No precipitation was observed in controls lacking HA-Nle or anti-HA (lanes 4, 6). Together these results indicate that Notchless binds directly to the intracellular domain of Notch.

204 F T O 0 8 2 0 6 6 0

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CLAIMS

- 1) A protein or fragment thereof comprising the sequence of SEQ ID No. 1, or a functional equivalent thereof.
- 2) A protein or fragment thereof according to claim 1 which regulates the activity of a protein product of a gene in the Notch gene family.
- 3) A protein or fragment thereof according to claim 2 which regulates the activity of Notch 1.
- 4) A protein or fragment thereof according to either of claims 2 or 3 wherein the Notch gene is mammalian, preferably human.
- 5) A protein or fragment thereof according to any preceding claim which has been altered by deletion, insertion, substitution, or mutation of one or more amino acids.
- 6) A protein or fragment thereof according to any preceding claim which is recombinant.
- 7) A protein or fragment thereof according to any preceding claim that binds specifically to a member of the Notch protein family, preferably to Notch 1.
- 8) A protein or fragment thereof according to any preceding claim that is associated with an enzyme effector or reporter molecule.
- 9) A protein or fragment thereof according to claim 8 that is associated with a protease enzyme.
- 10) A protein or fragment thereof according to any preceding claim that is genetically or chemically fused to one or more peptides or polypeptides.
- 11) A protein or fragment thereof according to any preceding claim attached to a label.
- 12) A therapeutic or diagnostic composition comprising a protein or fragment thereof according to any preceding claim.

13) A protein or fragment thereof according to any preceding claim or composition according to claim 12 for use in therapy.

14) A protein or fragment thereof according to any preceding claim which for use as a pharmaceutical.

5 15) Use of a protein or fragment thereof according to any preceding claim as a pharmaceutical.

16) The use of a protein or fragment thereof according to any of claims 1-11 in the manufacture of a medicament for the treatment or prevention of cancer, or of a neurodegenerative disease.

10 17) A process for the identification of a compound capable of modifying the levels of expression or activity of a Notch protein comprising screening a Notchless mutant in a sensitised Notch genetic background with a candidate compound and selecting for an altered phenotype of the Notchless mutant.

18) A compound identified by the process of claim 17.

15 19) A nucleic acid sequence comprising:

a) the sequence of SEQ ID No 2, or

b) a sequence that encodes on expression the amino acid sequence encoded by the sequence of part a), or

20 c) a fragment of the DNA sequence of part a) or part b) that comprises a nucleotide sequence that encodes the Notch binding domain.

20) The nucleic acid molecule of claim 19 which comprises DNA, cDNA or RNA.

21) A probe capable of screening for the Notchless gene, prepared from the nucleic acid sequence of claim 19.

25 22) A cloning or expression vector comprising a nucleic acid molecule according to any one of claims 19-21.

- 23) A host cell transformed or transfected with a nucleic acid according to any of claims 19-21 or with a vector according to claim 22.
- 24) A transgenic animal that has been transformed with a nucleic acid molecule according to any one of claims 19-21 or with a vector according to claim 22.
- 5 25) A method of preparing a protein or fragment thereof according to any of claims 1-11, comprising expressing a vector according to claim 22 in a host cell and culturing said host cell under conditions where said protein is expressed, and recovering said protein thus expressed.

1/10

FIG. 1

yeast
c.elegans
fly
mouse
human
frog

MSTLIPPPSKKQKKEAQLPREVAIIPKDLPNVSIKFOALDTGDNVGGALRV
-----PQISVSEDENELG---GSGILV
-----MQETDTEQEATPHTIQARLVYTGEEAGPPIDL
-----EEAAAGD-VQRLLVQFQDEGGQLLGSPFDV
-----GSPFDV
-----MKEDVGRLLIQFKNENGEGLGTPFDV
* : :

yeast
c.elegans
fly
mouse
human
frog

EELLNQLNGTSDDPVPYTFSCITIQGKKASDPVKTIDITDNLVSSLIKPGYN
QILCNQLLGSR---FCLNNEFSVSG-----AEIVDSIRKSLEEIDFE
GLICNALLKNE---EATPYLFFVGE-----DEIKKSLEDTLDLASVD
XLVCNALL-AQEEPLPLAFYVHD-----AEIVSSLGKTLESQSV-
QLVCNALL-AQEDPCPLAFFVHD-----AEIVSSLGKTLESQAV-
QLVCNALL-QEEDPVPLAFFVQD-----LEIVTSLDKTLEKQSV-
: * * : * : : *

yeast
c.elegans
fly
mouse
human
frog

YTPRAVFKVKPVTRSSSAIAGHGSTILCSAFAPHTSSRMVTGAGDNTARIW
YQPQAVFRVRPVTRCSASIPGHGEPVISAQFSPDGRG-LASGSGDQTMRIW
YQPQAVFKVRPVTRCTSSMPGHAEAVVSLNFSPDGAH-LASGSGDTTVRLW
YQPQAVFRVRAVTRCTSS-----
-QPQXLFVRVAVTRCTS-----
YQPQAVFKVRVAVTRCTSSLEGHTEAVISVAFSPTGKY-LASGSGDTTVRFW
* : : * : : * : : *

yeast
c.elegans
fly
mouse
human
frog

TLKGHYNWVLCVSWSPDGEVIATGSMDNTIRLWDPKSGQCLGDALRGHSHK
TCKSHKSWVLCIAWSPDATKIASACKNGEICIWNAKTGEQIGKTLKRHKQW
TCTGHKQWVLCVSWAPDGKRLASGCKAGSIIIWDPETGQQKGRPLSGHKKH

TSKGHTHWVLSIAWSPDGKKLASGCKNSQIFIWDPSTGKQIGKPLTGHSKW

yeast
c.elegans
fly
mouse
human
frog

LVKPGSKPRLASSSKDGTIKIWDTVSRVCQYTMSGHTNSVSCVKWGGQGLL
-----TVKMWR-----ADDGVMCRNMTG---
HRDPECR-KLASASGDGDCRIWDVKLGQCLMNIAGHTNAVTAVRWGGAGLI

HLNPESRY-LASASKDCTIRIWDTVMGQCQKILTSHTQSVTAVKWGGDGLL

yeast
c.elegans
fly
mouse
human
frog

RVWDINSQGRGINILKSHAHWVNHLSTDYALRIGAFDHTGKK-----PS
-----HAHWINTLALNTDYALRTSCFE-----PS
KMWR-AADGILCRTFSGHAWVNNIALSTDYVLRTPGFHPVKDRSKSHLSL

KAWR-AQDGVLCRTLQGHAWVNTMALSTDYVLRKGAFNPADAS--VNPQD

yeast
c.elegans
fly
mouse
human
frog

LENYEKICKKNGNSEEMMVTASDDYTMFLWNPLKSTKPIARMTGHQKLVNH
-----INRMTGHMQLVNO
LKRYQAVCP---DEVESLVSCSDNTLYLWRN-NQNKCVERTMTGHQNVND

EKALKRSNEVRGQGPRLVSGSEDFTLFLWAPAEKKPLQRMTHQALINE

yeast
c.elegans
fly
mouse
human
frog

IVSASFDNSIKLWDGRDGKFISTFRGHASVYQVAWSSDCRLLVSCSKDTT
IASASFDKSVKLVCGRTGKYLASFGRHVGPVYQVAWSADSRLLVSGSADST
IASASFDKSVRLWRASDGQYMATFRGHVQAVYTVAWSADSRLIVSGSKDST

IASASFDKSIKLWDGKTGKFLTSRGRHVSAVYQIAWSADSRLLVSGSSDST

yeast
c.elegans
fly
mouse
human
frog

KLSVDLPGIKTKLY-VDWSVDGKRVCSSGGKDKMVRLWTH
SLYYDLPGHGDEVFTVDWSPEGTKVVSAGGKDKVLKLW--
KLAQELPGHADEVFGVDWAPDGSRVASGGKDKVIKLWAY

KLLIDLPGHADEVYSVDWSPDGQRVASGGKDKCLRIWRK

2/10

FIG. 2(A)

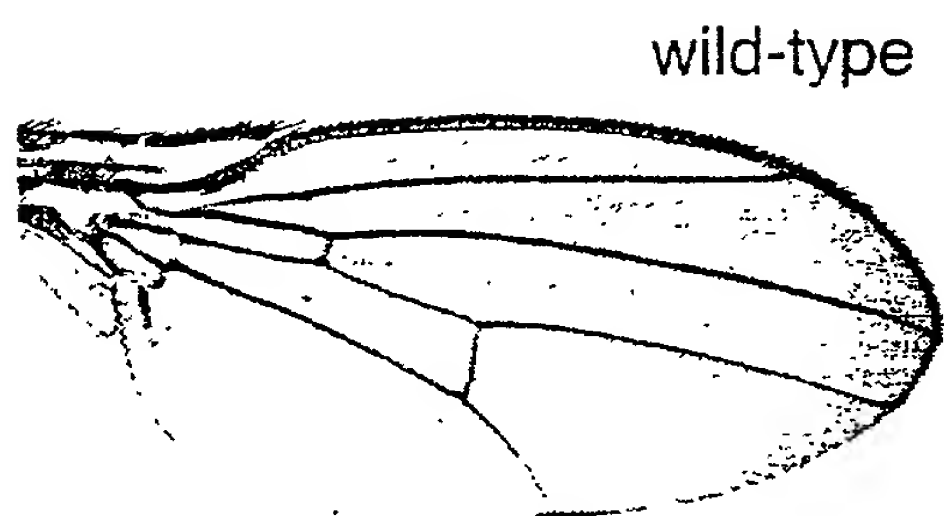


FIG. 2(B)



FIG. 2(C)

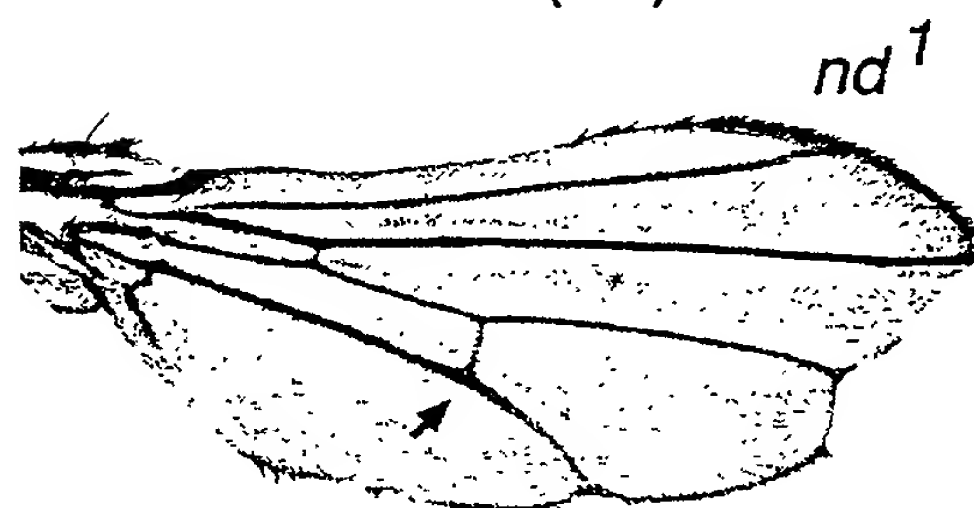


FIG. 2(D)

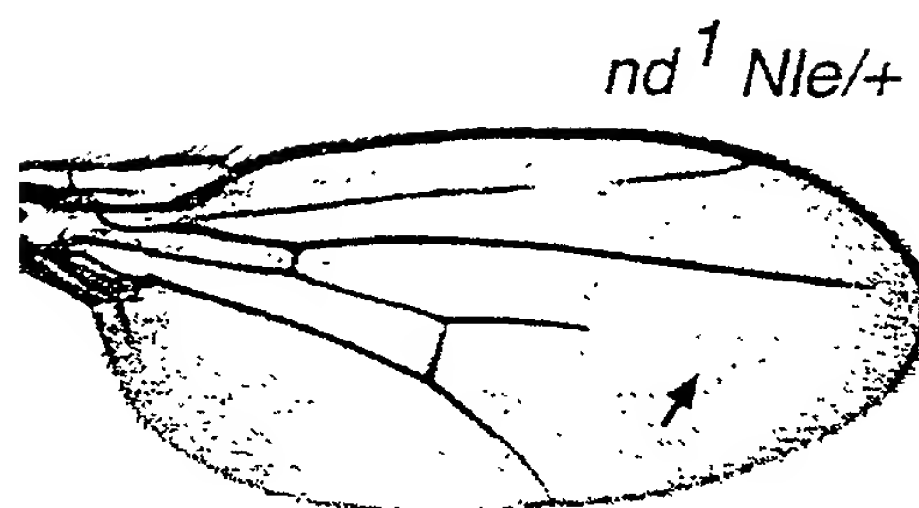
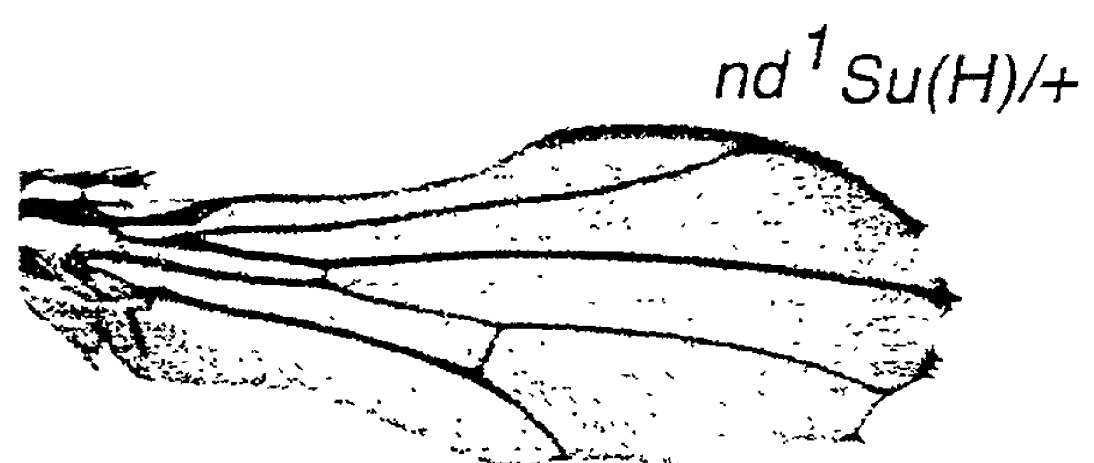


FIG. 2(E)



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FIG. 2(F)

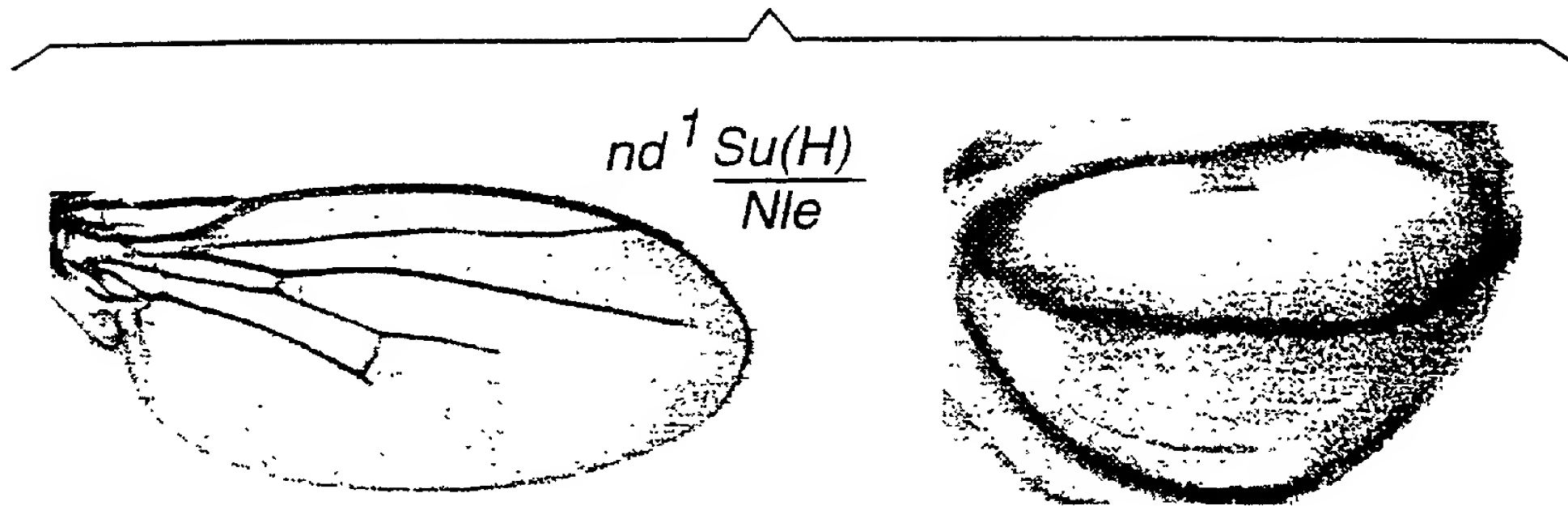


FIG. 2(G)

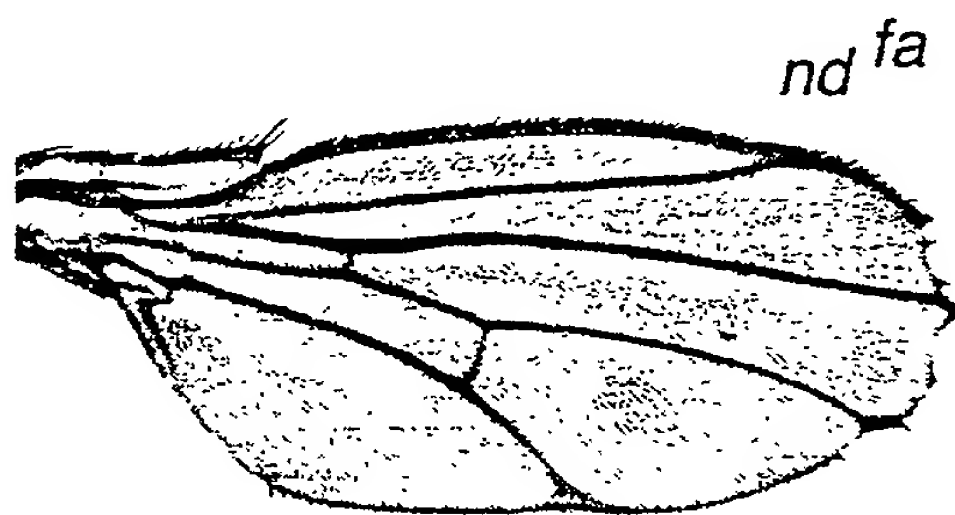
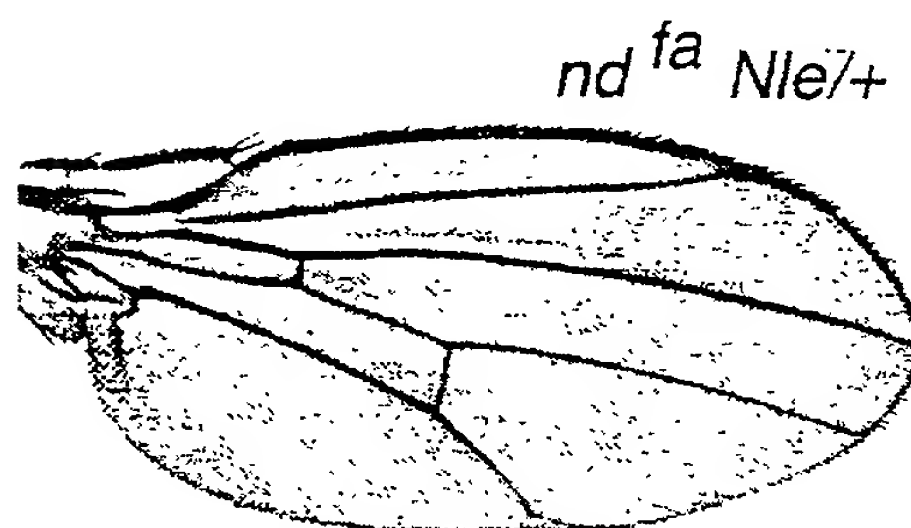


FIG. 2(H)



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FIG. 3(A)

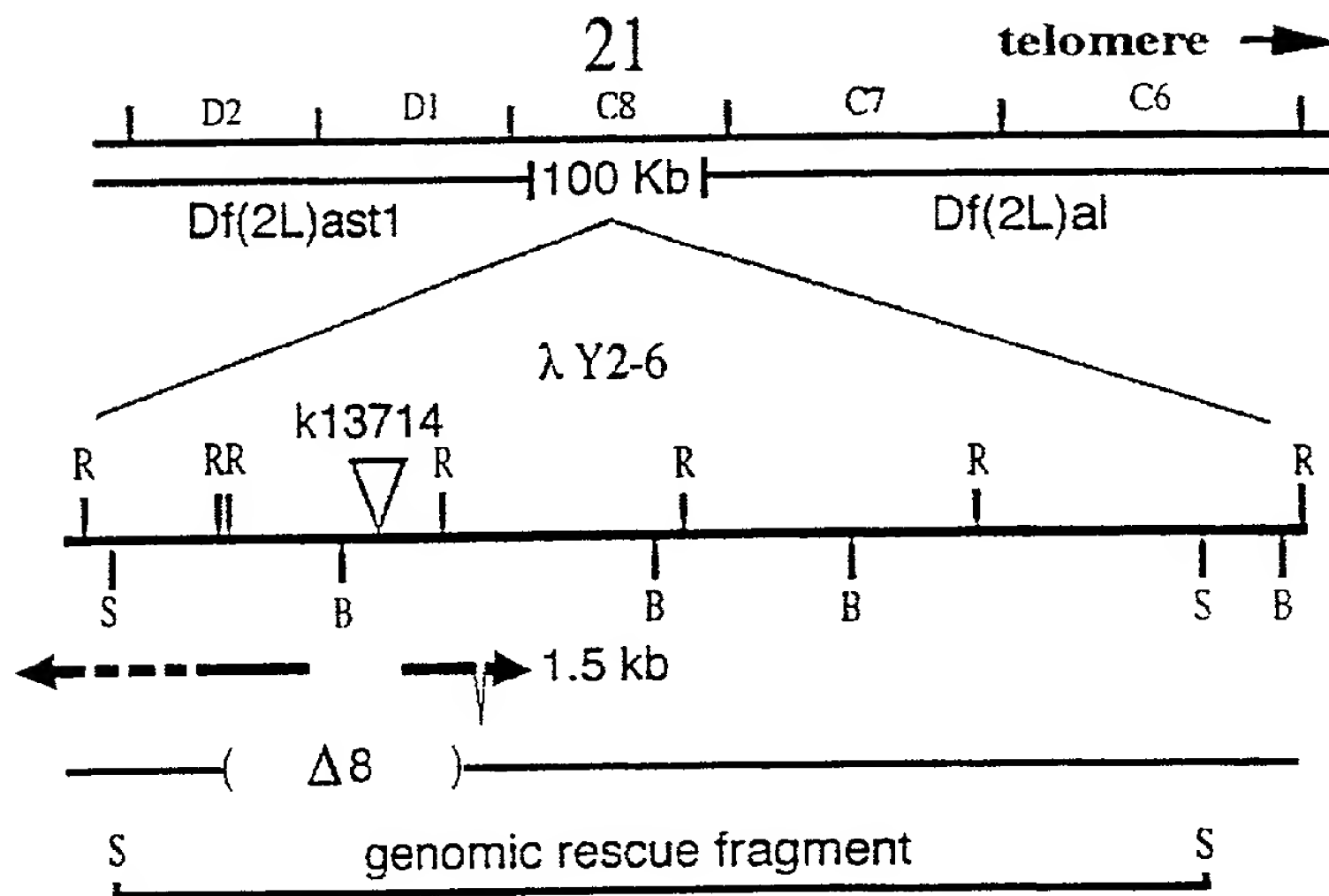


FIG. 3(B)

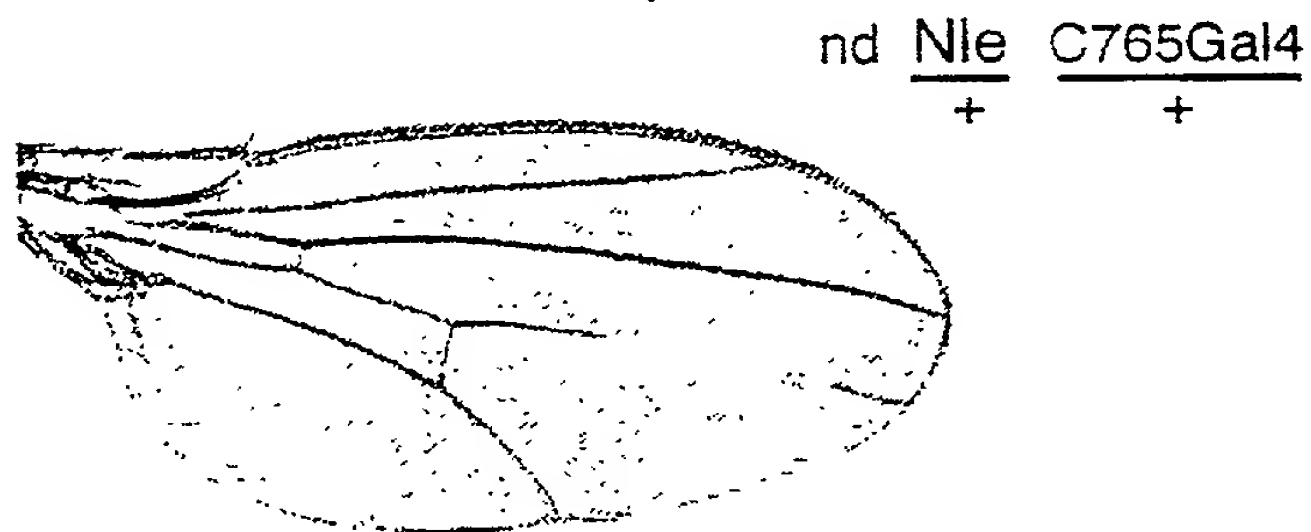
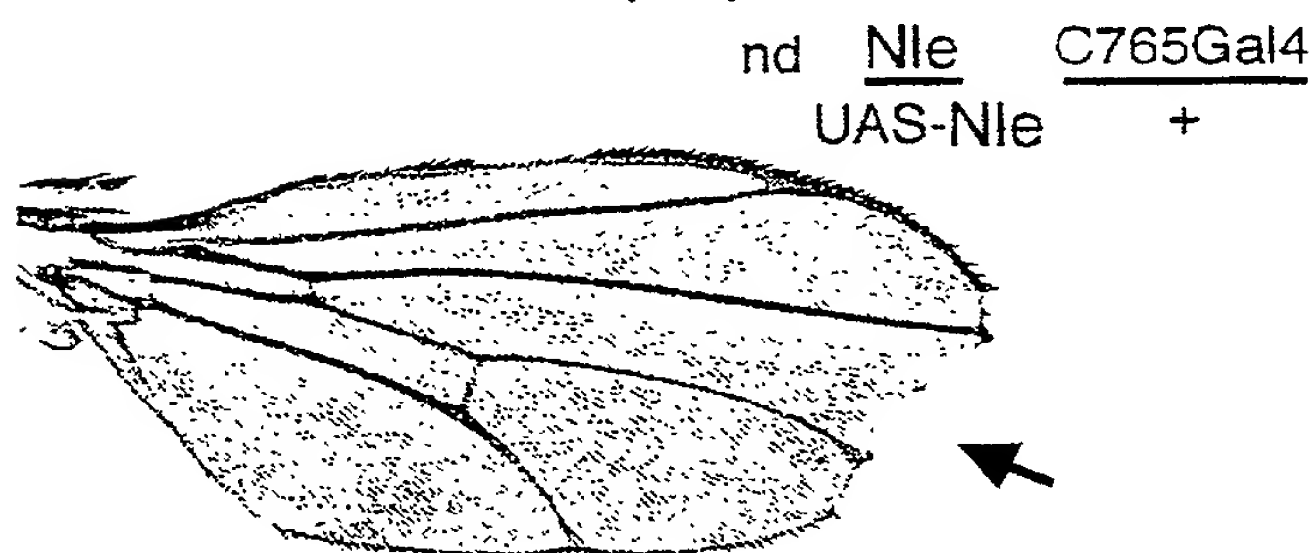


FIG. 3(C)



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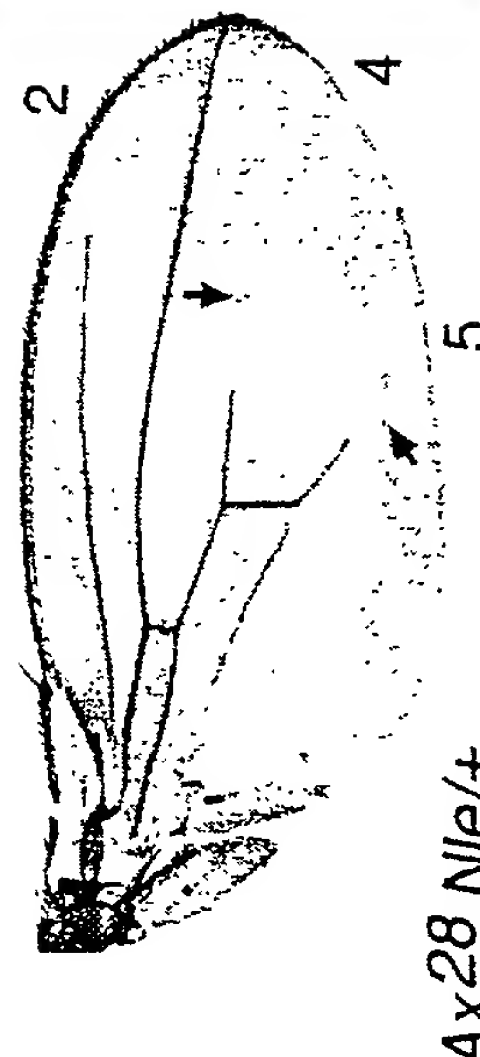
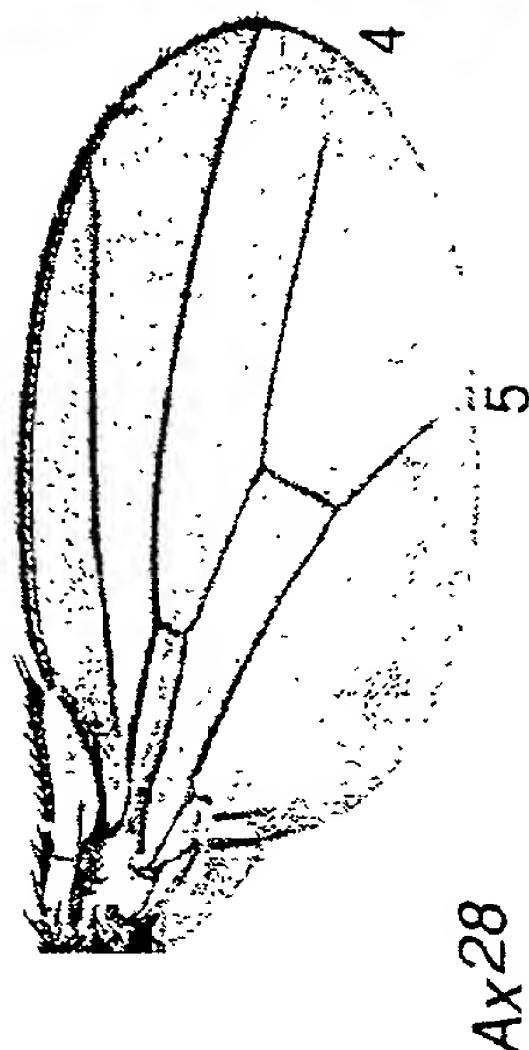
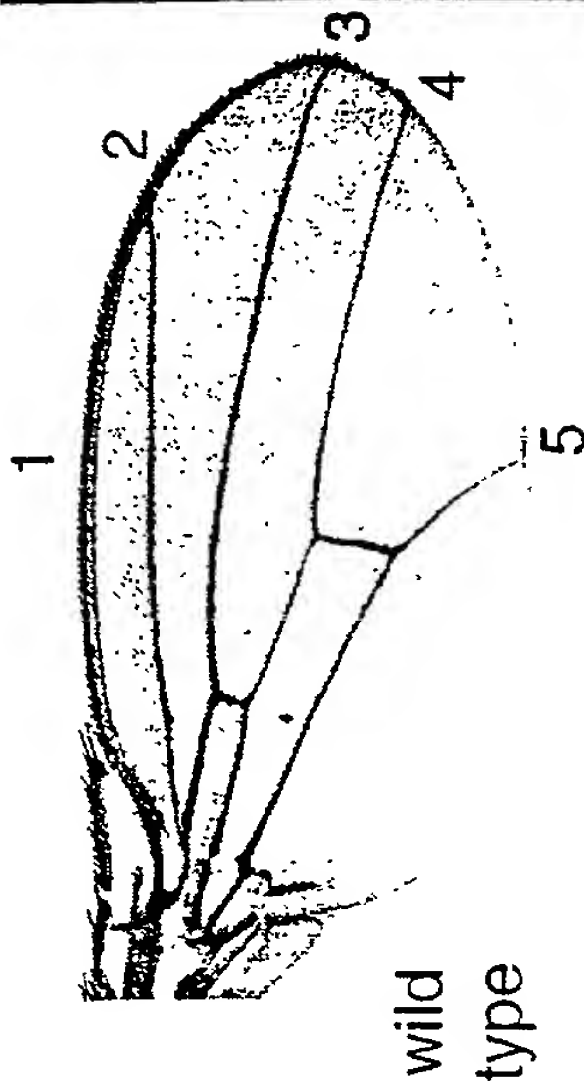
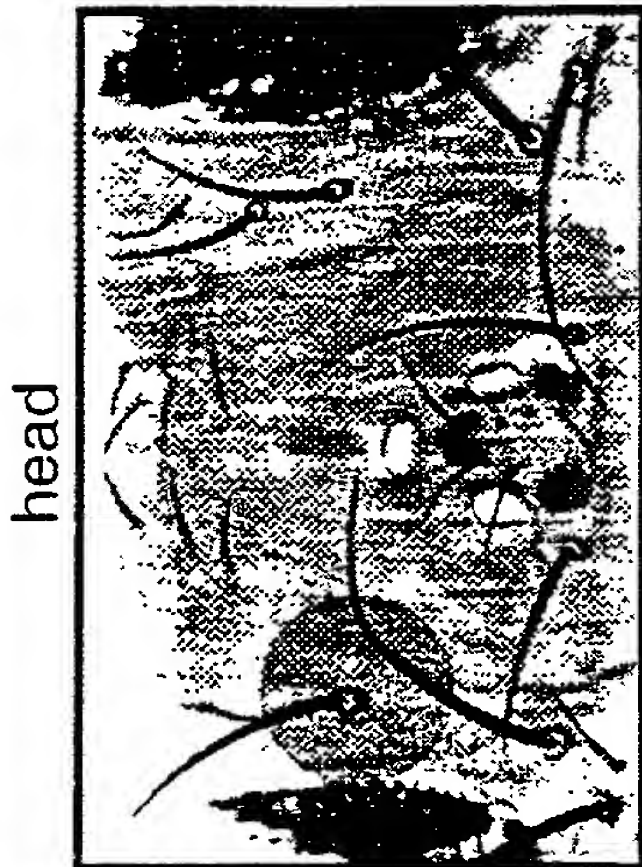


FIG. 4(A)

FIG. 4(B)

FIG. 4(C)

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FIG. 5(A)

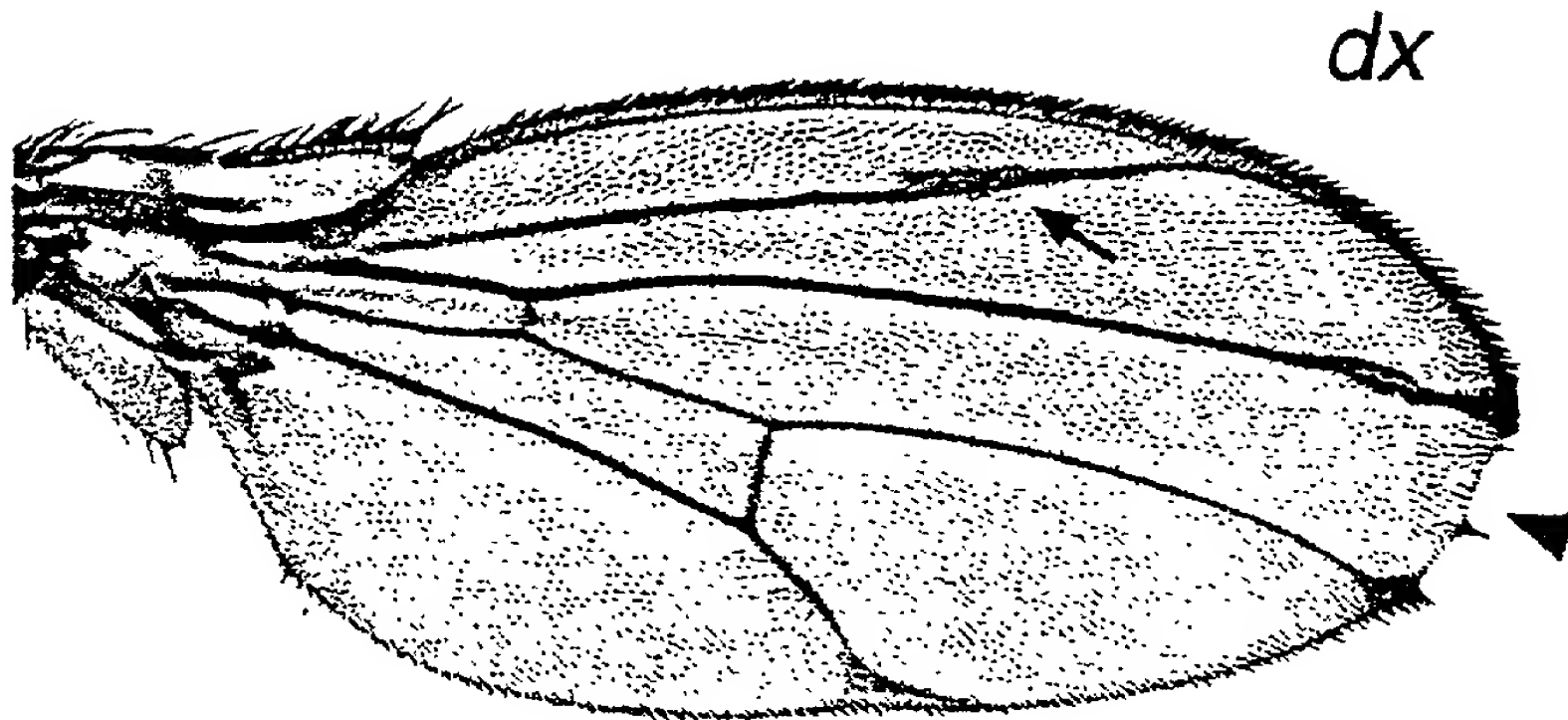
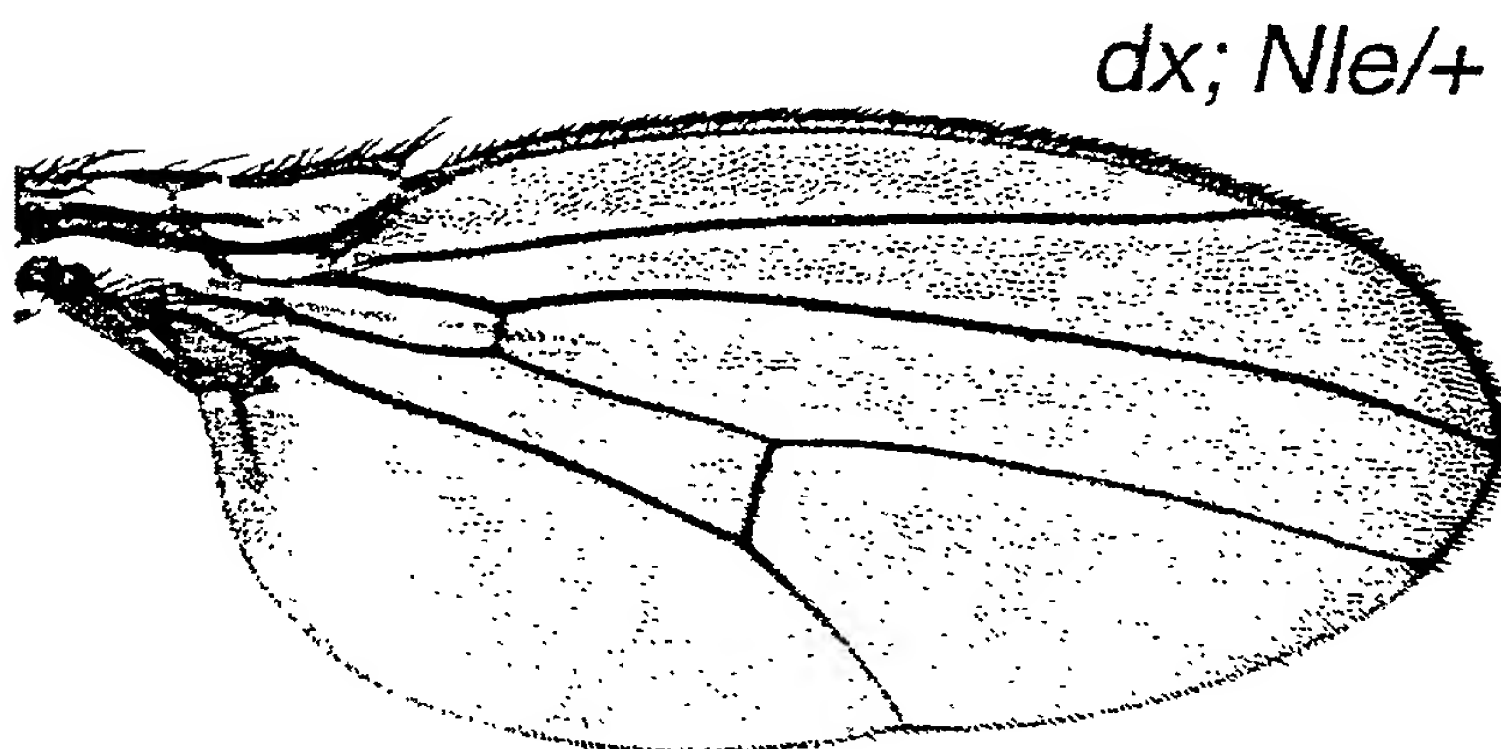


FIG. 5(B)



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FIG. 5(C)

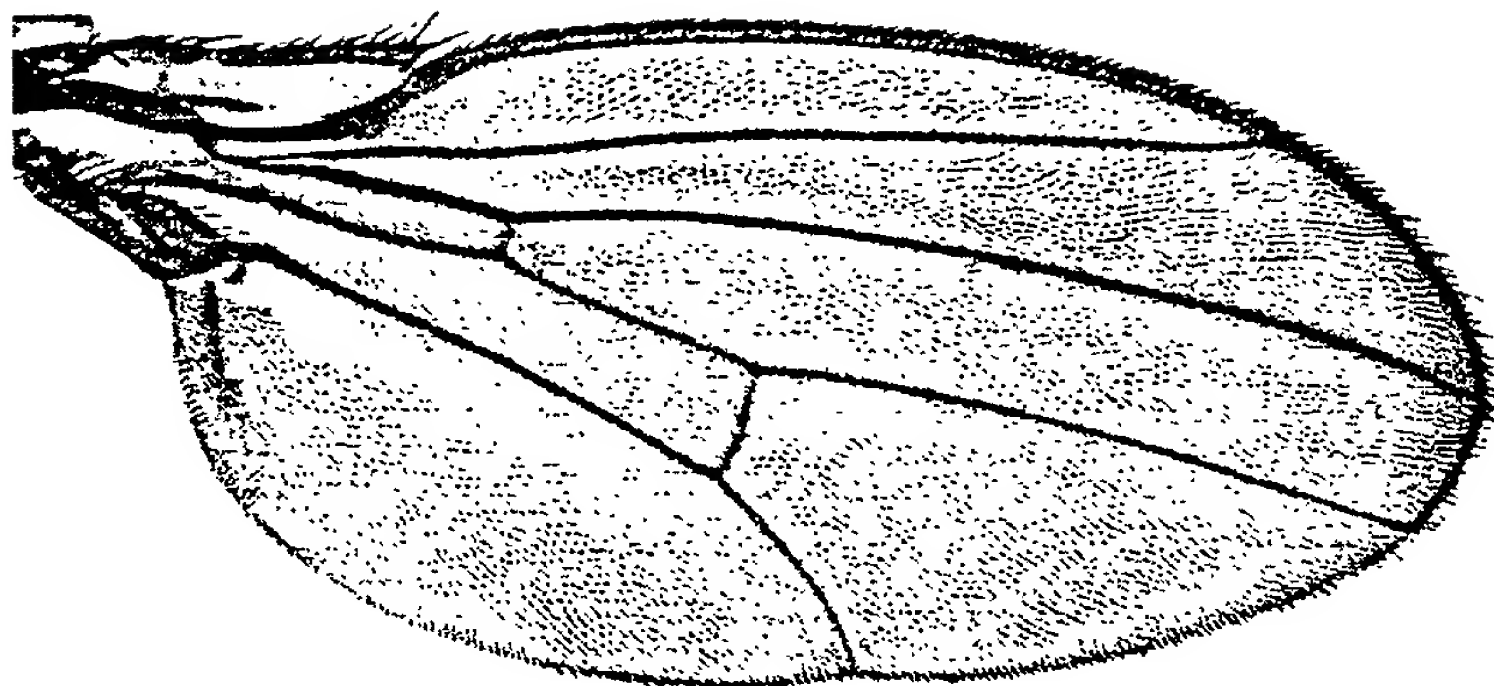
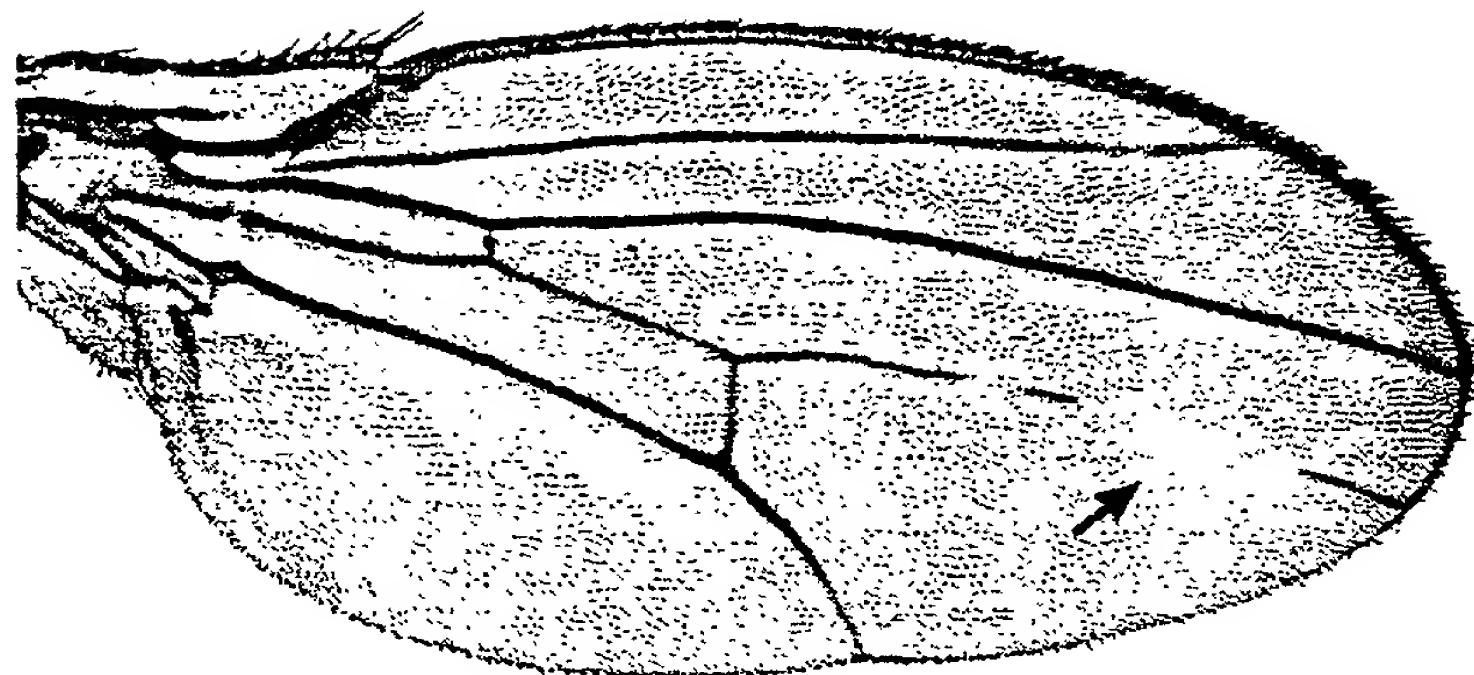
HS-dx

FIG. 5(D)

HS-dx; Nle/+

WD-40 repeats

[illegible]

FIG. 6(A)

Drosophila
Xenopus
C. elegans
S. cerevisiae
mouse
human

FIG. 6(B)

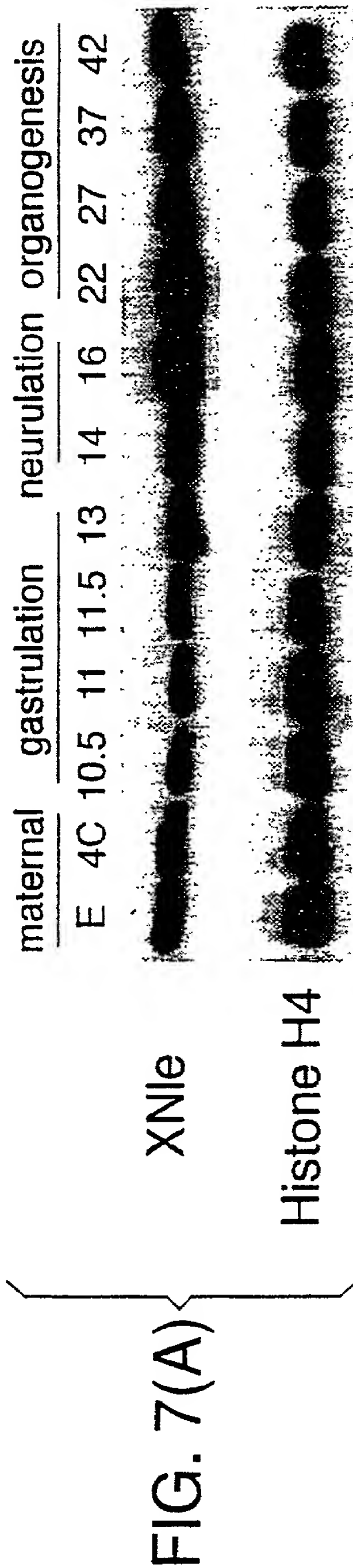
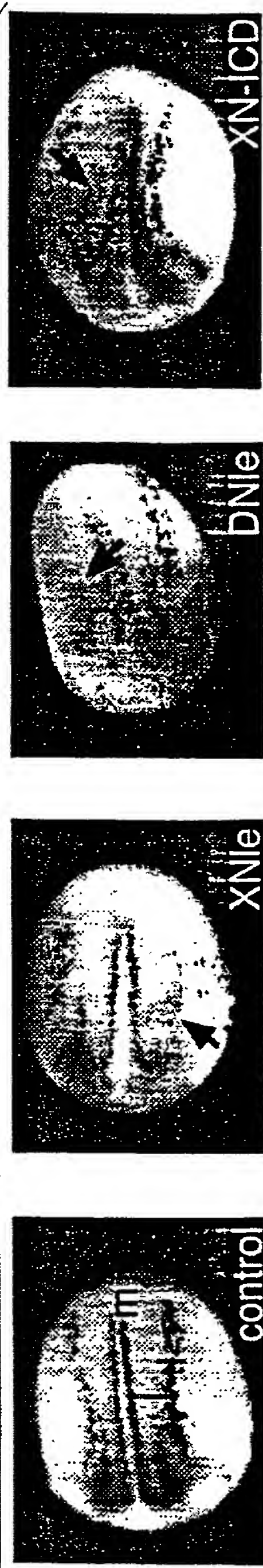


FIG. 7(B)



FIG. 7(C)



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FIG. 8(A)

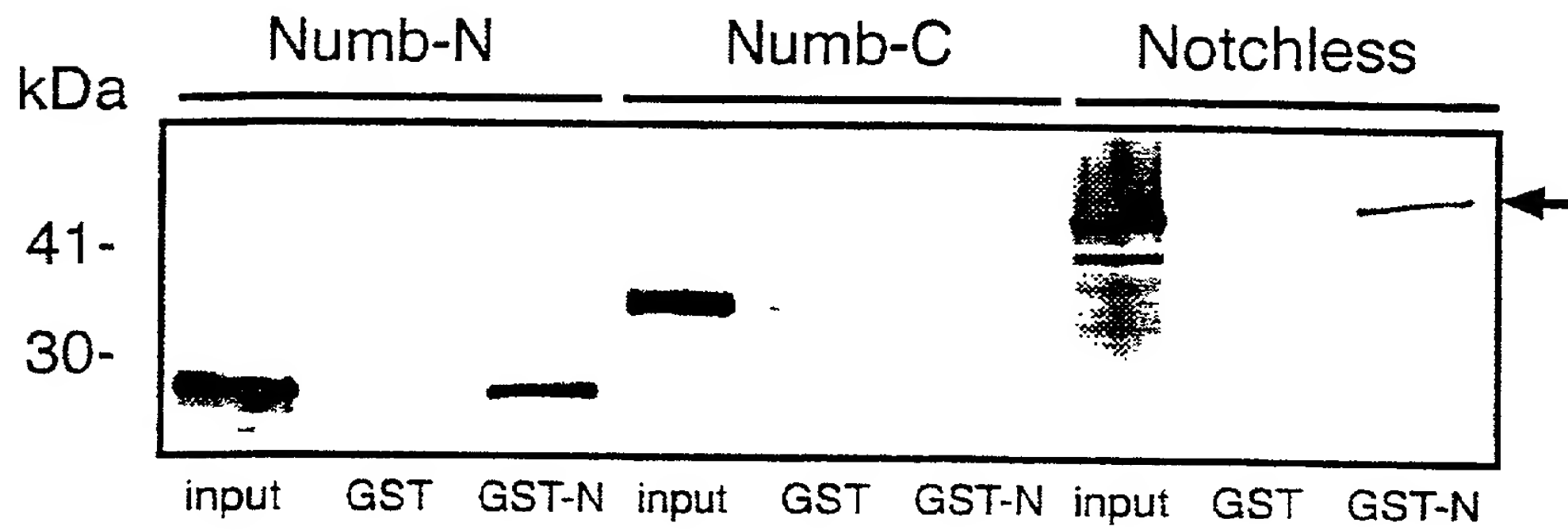
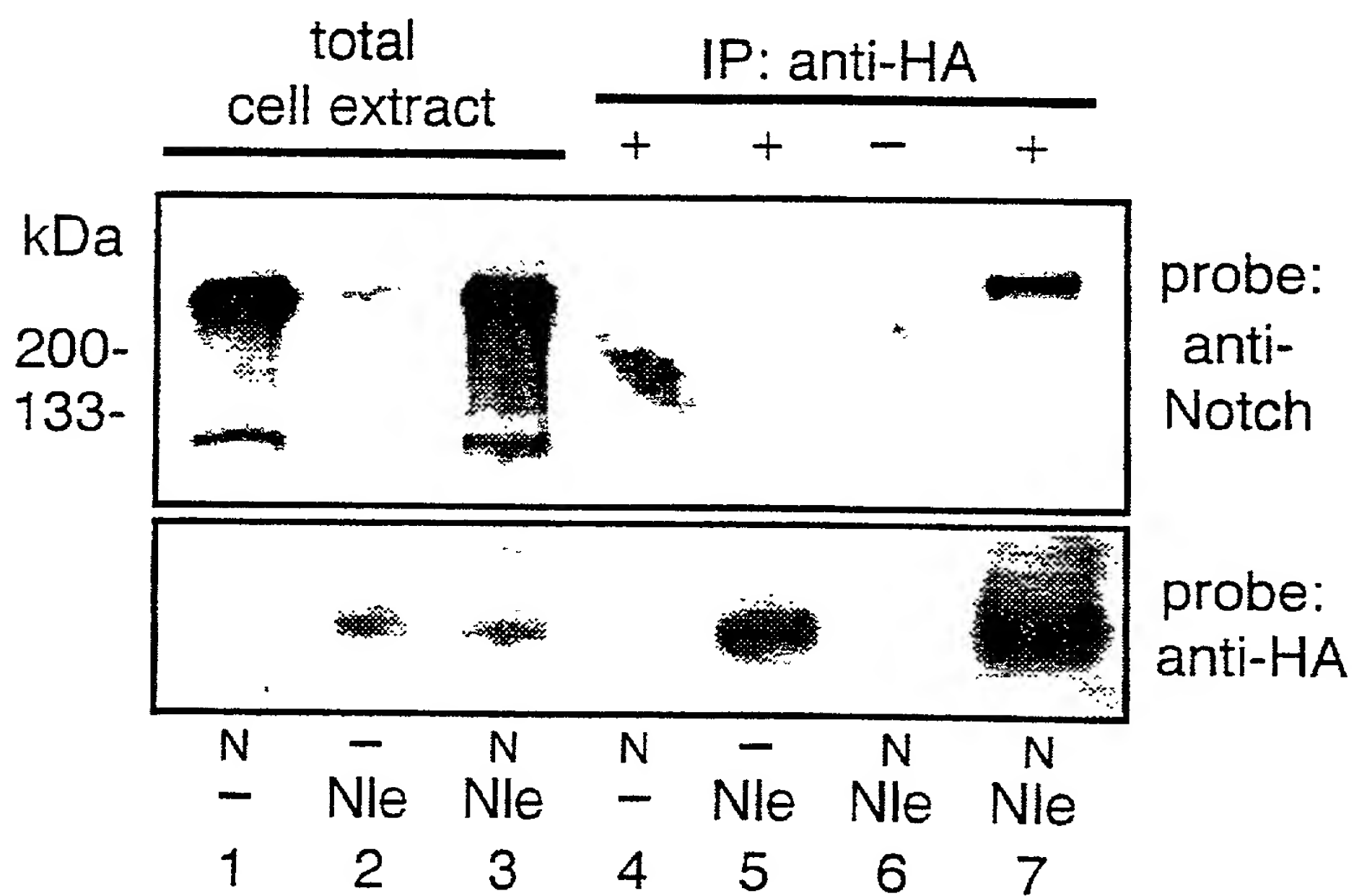


FIG. 8(B)



Sequence I.D. No. 1: *Drosophila* Notchless protein

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5 EAVVSLNFSPDGAHLASGSGD TTVRLWDLNTETPHFTCTGHKQWVLCVSWAP
DGKRLASGCKAGSIIIWDPETGQQKGRPLSGHKKHINCLAWEPYHRDPECRKL
ASASGDGDCRIWDVKLGQCLMNIAGHTNAVTA VRWGGAGLIYTSSKDRTVK
MWRAADGILCRTFSGHAHWVNNIALSTDYVLRTGPFHPVKDRSKSHLSLSTEE
LQESALKRYQAVCPDEVESLVSCSDDNTLYLWRNNQNK CVERMTGHQNVVN
10 DVKYSPDVKLIASASFDKSVRLWRASDGQYMATFRGHVQAVYTVAWSADSRL
IVSGSKDSTLKVWSVQTKKLAQELPGHADEVFGVDWAPDGSRVASGGKDKVI
KLWAY

Sequence I.D. No. 2: *Drosophila Nle* cDNA

15
aattcccaaaaaATGCAGGAGACGGACACGGAGCAAGAGGCCACGCCACATACGA
TACAGGCGCGCCTCGTTTACACGGGCGAGGAAGCCGGCCCGCCAATCGACC
TGCCGGCAGGAATCACTACCCAGCAATTGGGACTGATTTGCAACGCGCTGC
TGAAAAACGAGGAAGCCACTCCATATTTGTTTTTCGTGGGCGAGGATGAGA
20 TCAAGAAGAGCCTGGAGGACACGTTGGACTTGGCGTCAGTGGACACCGAAA
ACGTGATCGATATTGTGTATCAGCCACAGGCGGTTTTCAAAGTGCGCCCAGT
GACAAGATGCACGAGTTCCATGCCGGGACACGCCGAGGCTGTGGTTTTCGCT
GAATTTCAGCCCCGGATGGTGCTCATCTCGCCAGTGGAAGTGGCGACACCAC
AGTGCGATTGTGGGATCTTAACACAGAGACACCGCACTTCACCTGCACAGG
25 TCATAAGCAGTGGGTTCTGTGCGTATCCTGGGCTCCGGATGGCAAACGGTTG
GCCAGCGGTTGCAAAGCGGGCTCTATAATCATCTGGGACCCGGAGACGGGT
CAGCAGAAGGGGCGACCCTTGAGTGGGCACAAGAAACACATCAACTGCCTC
GCCTGGGAACCGTATCATCGCGATCCGGAGTGCAGGAAACTTGCTTCCGCC
AGTGGAGACGGGGACTGCCGGATTTGGGACGTAAAATTGGGCCAGTGCCTT

ATGAACATTGCCGGACACACAAATGCTGTGACAGCAGTGAGATGGGGGTGGA
GCGGGCCTTATTTATACATCCTCCAAAGATCGCACAGTGAAGATGTGGCGA
GCAGCTGATGGAATCTTGTGCCGGACGTTCTCTGGCCAAGCTCACTGGGTAA
ACAACATTGCGCTGAGCACCGATTACGTCCTGCGCACTGGTCCATTCCATCC
5 GGTGAAGGATCGCTCCAAGAGCCACCTCAGTTTGAGCACTGAGGAATTGCA
GGAATCTGCCTTGAAGCGCTACCAGGCCGTGTGCCCTGACGAGGTGGAGTC
GCTGGTTTCCTGTTCGGATGACAACACCCTCTATCTGTGGCGGAACAACCAG
AACAAGTGCGTTGAGCGCATGACAGGGCACCAGAACGTGGTCAACGATGTG
AAATATTCGCCGGATGTAAAGCTAATTGCGTCTGCTTCATTTGACAAGTCAG
10 TGCGTCTGTGGCGAGCCAGCGATGGTCAGTACATGGCCACCTTCCGGGGTC
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TTCCGGCAGCAAAGACTCAACTCTAAAAGTATGGAGTGTGCAGACGAAGAA
ACTGGCACAGGAGCTGCCTGGACATGCGGATGAGGTGTTCGGAGTGGACTG
GGCGCCCGATGGCTCTAGAGTTGCCTCTGGTGGCAAGGACAAAGTTATAAA
15 GCTATGGGCTTATTAAcaaatcattaactgtacacggtgaagaaaatacttaggaataaagtaaaacgtcctgag
taaa

Pot

SIUS

P020269US

DIKE, BRONSTEIN, ROBERTS & CUSHMAN
INTELLECTUAL PROPERTY GROUP OF
EDWARDS & ANGELL, LLP
P.O. Box No. 9169
Boston, Massachusetts 02209

Docket No. 71745/55880

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed at 201) below or an original, first and joint inventor (if plural names are listed at 201-206 below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **REGULATOR OF NOTCH SIGNALING ACTIVITY**

which is described and claimed in:

- ☐ the specification attached hereto.
- ☐ the specification in U.S. Application Serial Number 09/830,980, filed on May 2, 2001.
- ☐ the specification in PCT international application Number, filed on _____; and was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign/PCT Applications and Any Priority Claims Under 35 U.S.C. §119:			
Application No.	Filing Date	Country	Priority Claimed Under 35 U.S.C. §119?
9824045.0	3 November 1999	GB	X YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S. Applications or PCT International Applications Designating the U.S-Benefit Under 35 U.S.C. §120

U.S. Applications		Status (Check One)		
Application Serial No.	U.S. Filing Date	Patented	Pending	Abandoned
PCT Applications Designating the U.S.				
Application No.	Filing Date	U.S. Serial No. Assigned		
PCT/IB99/01891	11/03/99	09/830,980		X

**CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. §119(e))**

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Applicant	Provisional Application Number	Filing Date

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

David G. Conlin (Reg. No. 27,026)
 George W. Neuner (Reg. No. 26,964)
 Linda M. Buckley (Reg. No. 31,003)
 Peter J. Manus (Reg. No. 26,766)
 Peter F. Corless (Reg. No. 33,860)

Cara Z. Lowen (Reg. No. 38,227)
 William J. Daley, Jr. (Reg. No. 35,487)
 David A. Tucker (Reg. No. 27,840)
 Robert L. Buchanan (Reg. No. 40,927)
 Christine C. O'Day (Reg. No. 38,256)

Lisa Swiszc Hazzard (Reg. No. 44,368)
 George W. Hartnell, III (Reg. No. 42,639)
 Steven M. Jensen (Reg. No. 42,693)
 Kathryn A. Piffat (Reg. No. 34,901)

SEND CORRESPONDENCE TO:

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 Intellectual Property Practice Group
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 P.O. Box 9169
 Boston, Massachusetts 02109

DIRECT TELEPHONE CALLS TO:

Cara Z. Lowen
 (617) 439-4444

201	FULL NAME OF INVENTOR	LAST NAME Cohen	FIRST NAME Stephen	MIDDLE NAME
	RESIDENCE			CITIZENSHIP
	POST OFFICE ADDRESS			
202	FULL NAME OF INVENTOR	LAST NAME Bouwmeester	FIRST NAME Antonius	MIDDLE NAME
	RESIDENCE			CITIZENSHIP
	POST OFFICE ADDRESS			
203	FULL NAME OF INVENTOR	LAST NAME Royet	FIRST NAME Julien	MIDDLE NAME
	RESIDENCE			CITIZENSHIP
	POST OFFICE ADDRESS			

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201	Signature of Inventor 202
Date:	Date:
Signature of Inventor 203	
Date:	

170286

FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	Cohen	Stephen	<i>Michael</i>
RESIDENCE	<i>Kleinschmidtstr 60 HEIDELBERG 69115 DEU</i>		CITIZENSHIP
			<i>CANADIAN</i>
POST OFFICE ADDRESS			
FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	Bouwmeester	Antonius	
RESIDENCE			CITIZENSHIP
POST OFFICE ADDRESS			
FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	Royet	Julien	
RESIDENCE			CITIZENSHIP
POST OFFICE ADDRESS			

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201	Signature of Inventor 202
Date: <i>Stephen Cohen</i> 03.01.02	Date:
Signature of Inventor 203	
Date:	

SIUS PO20269US

FULL NAME OF INVENTOR	LAST NAME Cohen	FIRST NAME Stephen	MIDDLE NAME
RESIDENCE			CITIZENSHIP
POST OFFICE ADDRESS			
FULL NAME OF INVENTOR	LAST NAME Bouwmeester	FIRST NAME Antonius	MIDDLE NAME Johannes M.
RESIDENCE	Bergstrasse 1 69120 Heidelberg GDEX Germany		CITIZENSHIP Dutch
POST OFFICE ADDRESS	Meyerkhofstrasse 1 69117 Heidelberg Germany		
FULL NAME OF INVENTOR	LAST NAME Royet	FIRST NAME Julien	MIDDLE NAME
RESIDENCE			CITIZENSHIP
POST OFFICE ADDRESS			

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201	Signature of Inventor 202
Date:	Date: 07-12-01
Signature of Inventor 203	
Date:	

170286

SIUS PO20269 US

FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	Cohen	Stephen	
RESIDENCE			CITIZENSHIP
POST OFFICE ADDRESS			
FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	Bouwmeester	Antonius	
RESIDENCE			CITIZENSHIP
POST OFFICE ADDRESS			
FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	Royet	Julien	
RESIDENCE	18 rue Pablo Neruda 67540 Ostwald France FRX		French.
POST OFFICE ADDRESS	18 rue Pablo Neruda 67540 Ostwald France		

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor 201	Signature of Inventor 202
Date:	Date:
Signature of Inventor 203 <i>(Royet)</i>	
Date: 13/12/2001	

110286

SEQUENCE LISTING

<110> COHEN, STEPHEN
BOUWMEESTER, ANTONIUS
ROYET, JULIEN

<120> REGULATOR OF NOTCH SIGNALING ACTIVITY

<130> 55880(71745)

<140> 09/830,980

<141> 2001-05-02

<150> PCT/IB99/01891

<151> 1999-11-03

<150> GB 9824045.0

<151> 1998-11-03

<160> 16

<170> PatentIn Ver. 2.1

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<213> Drosophila sp.

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Pro Ala Gly Ile Thr Thr Gln Gln Leu Gly Leu Ile Cys Asn Ala Leu
35 40 45

Leu Lys Asn Glu Glu Ala Thr Pro Tyr Leu Phe Phe Val Gly Glu Asp
50 55 60

Glu Ile Lys Lys Ser Leu Glu Asp Thr Leu Asp Leu Ala Ser Val Asp
65 70 75 80

Thr Glu Asn Val Ile Asp Ile Val Tyr Gln Pro Gln Ala Val Phe Lys
85 90 95

Val Arg Pro Val Thr Arg Cys Thr Ser Ser Met Pro Gly His Ala Glu
100 105 110

Ala Val Val Ser Leu Asn Phe Ser Pro Asp Gly Ala His Leu Ala Ser
115 120 125

Gly Ser Gly Asp Thr Thr Val Arg Leu Trp Asp Leu Asn Thr Glu Thr
130 135 140

Pro His Phe Thr Cys Thr Gly His Lys Gln Trp Val Leu Cys Val Ser
 145 150 155 160
 Trp Ala Pro Asp Gly Lys Arg Leu Ala Ser Gly Cys Lys Ala Gly Ser
 165 170 175
 Ile Ile Ile Trp Asp Pro Glu Thr Gly Gln Gln Lys Gly Arg Pro Leu
 180 185 190
 Ser Gly His Lys Lys His Ile Asn Cys Leu Ala Trp Glu Pro Tyr His
 195 200 205
 Arg Asp Pro Glu Cys Arg Lys Leu Ala Ser Ala Ser Gly Asp Gly Asp
 210 215 220
 Cys Arg Ile Trp Asp Val Lys Leu Gly Gln Cys Leu Met Asn Ile Ala
 225 230 235 240
 Gly His Thr Asn Ala Val Thr Ala Val Arg Trp Gly Gly Ala Gly Leu
 245 250 255
 Ile Tyr Thr Ser Ser Lys Asp Arg Thr Val Lys Met Trp Arg Ala Ala
 260 265 270
 Asp Gly Ile Leu Cys Arg Thr Phe Ser Gly His Ala His Trp Val Asn
 275 280 285
 Asn Ile Ala Leu Ser Thr Asp Tyr Val Leu Arg Thr Gly Pro Phe His
 290 295 300
 Pro Val Lys Asp Arg Ser Lys Ser His Leu Ser Leu Ser Thr Glu Glu
 305 310 315 320
 Leu Gln Glu Ser Ala Leu Lys Arg Tyr Gln Ala Val Cys Pro Asp Glu
 325 330 335
 Val Glu Ser Leu Val Ser Cys Ser Asp Asp Asn Thr Leu Tyr Leu Trp
 340 345 350
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 355 360 365
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 Ala Ser Phe Asp Lys Ser Val Arg Leu Trp Arg Ala Ser Asp Gly Gln
 385 390 395 400
 Tyr Met Ala Thr Phe Arg Gly His Val Gln Ala Val Tyr Thr Val Ala
 405 410 415
 Trp Ser Ala Asp Ser Arg Leu Ile Val Ser Gly Ser Lys Asp Ser Thr
 420 425 430
 Leu Lys Val Trp Ser Val Gln Thr Lys Lys Leu Ala Gln Glu Leu Pro
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gcttccgcca gtggagacgg ggactgccgg atttgggacg taaaattggg ccagtgcctt 720
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gtggactggg cgcggatgg ctctagagtt gcctctgggt gcaaggacaa agttataaag 1440
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taaacgtcc tgagtaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaa 1555

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<210> 3

<211> 513

<212> PRT

<213> Saccharomyces cerevisiae

<400> 3

Met Ser Thr Leu Ile Pro Pro Pro Ser Lys Lys Gln Lys Lys Glu Ala
1 5 10 15

Gln Leu Pro Arg Glu Val Ala Ile Ile Pro Lys Asp Leu Pro Asn Val
20 25 30

Ser Ile Lys Phe Gln Ala Leu Asp Thr Gly Asp Asn Val Gly Gly Ala
35 40 45

Leu Arg Val Pro Gly Ala Ile Ser Glu Lys Gln Leu Glu Glu Leu Leu
 50 55 60
 Asn Gln Leu Asn Gly Thr Ser Asp Asp Pro Val Pro Tyr Thr Phe Ser
 65 70 75 80
 Cys Thr Ile Gln Gly Lys Lys Ala Ser Asp Pro Val Lys Thr Ile Asp
 85 90 95
 Ile Thr Asp Asn Leu Tyr Ser Ser Leu Ile Lys Pro Gly Tyr Asn Ser
 100 105 110
 Thr Glu Asp Gln Ile Thr Leu Leu Tyr Thr Pro Arg Ala Val Phe Lys
 115 120 125
 Val Lys Pro Val Thr Arg Ser Ser Ser Ala Ile Ala Gly His Gly Ser
 130 135 140
 Thr Ile Leu Cys Ser Ala Phe Ala Pro His Thr Ser Ser Arg Met Val
 145 150 155 160
 Thr Gly Ala Gly Asp Asn Thr Ala Arg Ile Trp Asp Cys Asp Thr Gln
 165 170 175
 Thr Pro Met His Thr Leu Lys Gly His Tyr Asn Trp Val Leu Cys Val
 180 185 190
 Ser Trp Ser Pro Asp Gly Glu Val Ile Ala Thr Gly Ser Met Asp Asn
 195 200 205
 Thr Ile Arg Leu Trp Asp Pro Lys Ser Gly Gln Cys Leu Gly Asp Ala
 210 215 220
 Leu Arg Gly His Ser Lys Trp Ile Thr Ser Leu Ser Trp Glu Pro Ile
 225 230 235 240
 Leu Val Lys Pro Gly Ser Lys Pro Arg Leu Ala Ser Ser Ser Lys Asp
 245 250 255
 Gly Thr Ile Lys Ile Trp Asp Thr Val Ser Arg Val Cys Gln Tyr Thr
 260 265 270
 Met Ser Gly His Thr Asn Ser Val Ser Cys Val Lys Trp Gly Gly Gln
 275 280 285
 Gly Leu Leu Tyr Ser Gly Ser His Asp Arg Thr Val Arg Val Trp Asp
 290 295 300
 Ile Asn Ser Gln Gly Arg Cys Ile Asn Ile Leu Lys Ser His Ala His
 305 310 315 320
 Trp Val Asn His Leu Ser Leu Ser Thr Asp Tyr Ala Leu Arg Ile Gly
 325 330 335
 Ala Phe Asp His Thr Gly Lys Lys Pro Ser Thr Pro Glu Glu Ala Gln
 340 345 350

Lys Lys Ala Leu Glu Asn Tyr Glu Lys Ile Cys Lys Lys Asn Gly Asn
 355 360 365

Ser Glu Glu Met Met Val Thr Ala Ser Asp Asp Tyr Thr Met Phe Leu
 370 375 380

Trp Asn Pro Leu Lys Ser Thr Lys Pro Ile Ala Arg Met Thr Gly His
 385 390 395 400

Gln Lys Leu Val Asn His Val Ala Phe Ser Pro Asp Gly Arg Tyr Ile
 405 410 415

Val Ser Ala Ser Phe Asp Asn Ser Ile Lys Leu Trp Asp Gly Arg Asp
 420 425 430

Gly Lys Phe Ile Ser Thr Phe Arg Gly His Ile Ala Ser Val Tyr Gln
 435 440 445

Val Ala Trp Ser Ser Asp Cys Arg Leu Leu Val Ser Cys Ser Lys Asp
 450 455 460

Thr Thr Leu Lys Val Trp Asp Val Arg Thr Arg Lys Leu Ser Val Asp
 465 470 475 480

Leu Pro Gly Ile Lys Thr Lys Leu Tyr Val Asp Trp Ser Val Asp Gly
 485 490 495

Lys Arg Val Cys Ser Gly Gly Lys Asp Lys Met Val Arg Leu Trp Thr
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His

<210> 4

<211> 351

<212> PRT

<213> Codonanthus elegans

<220>

<221> MOD_RES

<222> (184)..(185)

<223> Variable amino acid

<400> 4

Pro Gln Ile Ser Val Ser Glu Asp Glu Asn Glu Leu Gly Gly Ser Gly
 1 5 10 15

Ile Leu Val Pro Val Asp Ile Ser Thr Asn Glu Leu Gln Ile Leu Cys
 20 25 30

Asn Gln Leu Leu Gly Ser Arg Phe Cys Leu Asn Asn Glu Phe Ser Val
 35 40 45

Ser Gly Ala Glu Ile Val Asp Ser Ile Arg Lys Ser Leu Glu Glu Ile
 50 55 60

Asp Phe Glu Thr Leu Lys Leu Val Tyr Gln Pro Gln Ala Val Phe Arg
 65 70 75 80
 Val Arg Pro Val Thr Arg Cys Ser Ala Ser Ile Pro Gly His Gly Glu
 85 90 95
 Pro Val Ile Ser Ala Gln Phe Ser Pro Asp Gly Arg Gly Leu Ala Ser
 100 105 110
 Gly Ser Gly Asp Gln Thr Met Arg Ile Trp Asp Ile Glu Leu Glu Leu
 115 120 125
 Pro Leu His Thr Cys Lys Ser His Lys Ser Trp Val Leu Cys Ile Ala
 130 135 140
 Trp Ser Pro Asp Ala Thr Lys Ile Ala Ser Ala Cys Lys Asn Gly Glu
 145 150 155 160
 Ile Cys Ile Trp Asn Ala Lys Thr Gly Glu Gln Ile Gly Lys Thr Leu
 165 170 175
 Lys Arg His Lys Gln Trp Ile Xaa Xaa Leu Ala Trp Gln Pro Thr Val
 180 185 190
 Lys Met Trp Arg Ala Asp Asp Gly Val Met Cys Arg Asn Met Thr Gly
 195 200 205
 His Ala His Trp Ile Asn Thr Leu Ala Leu Asn Thr Asp Tyr Ala Leu
 210 215 220
 Arg Thr Ser Cys Phe Glu Pro Ser Lys Ile Asn Arg Met Thr Gly His
 225 230 235 240
 Met Gln Leu Val Asn Gln Val Val Phe Ser Pro Asp Thr Arg Tyr Ala
 245 250 255
 Ser Ala Ser Phe Asp Lys Ser Val Lys Leu Trp Cys Gly Arg Thr Gly
 260 265 270
 Lys Tyr Leu Ala Ser Phe Arg Gly His Val Gly Pro Val Tyr Gln Val
 275 280 285
 Ala Trp Ser Ala Asp Ser Arg Leu Leu Val Ser Gly Ser Ala Asp Ser
 290 295 300
 Thr Leu Lys Val Phe Glu Leu Lys Thr Lys Ser Leu Tyr Tyr Asp Leu
 305 310 315 320
 Pro Gly His Gly Asp Glu Val Phe Thr Val Asp Trp Ser Pro Glu Gly
 325 330 335
 Thr Lys Val Val Ser Gly Gly Lys Asp Lys Val Leu Lys Leu Trp
 340 345 350

<210> 5

<211> 103

<212> PRT
 <213> Mus sp.

<220>
 <221> MOD_RES
 <222> (39)
 <223> Variable amino acid

<400> 5
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 1 5 10 15
 Asp Glu Gly Gly Gln Leu Leu Gly Ser Pro Phe Asp Val Pro Val Asp
 20 25 30
 Ile Thr Pro Asp Lys Leu Xaa Leu Val Cys Asn Ala Leu Leu Ala Gln
 35 40 45
 Glu Glu Pro Leu Pro Leu Ala Phe Tyr Val His Asp Ala Glu Ile Val
 50 55 60
 Ser Ser Leu Gly Lys Thr Leu Glu Ser Gln Ser Val Glu Thr Glu Lys
 65 70 75 80
 Ile Val Asp Ile Ile Tyr Gln Pro Gln Ala Val Phe Arg Val Arg Ala
 85 90 95
 Val Thr Arg Cys Thr Ser Ser
 100

<210> 6
 <211> 78
 <212> PRT
 <213> Homo sapiens

<220>
 <221> MOD_RES
 <222> (66)
 <223> Variable amino acid

<400> 6
 Gly Ser Pro Phe Asp Val Pro Val Asp Ile Thr Pro Asp Arg Leu Gln
 1 5 10 15
 Leu Val Cys Asn Ala Leu Leu Ala Gln Glu Asp Pro Cys Pro Leu Ala
 20 25 30
 Phe Phe Val His Asp Ala Glu Ile Val Ser Ser Leu Gly Lys Thr Leu
 35 40 45
 Glu Ser Gln Ala Val Glu Thr Glu Lys Val Leu Asp Ile Tyr Gln Pro
 50 55 60
 Gln Xaa Leu Phe Arg Val Arg Ala Val Thr Arg Cys Thr Ser
 65 70 75

<213> Xenopus laevis

Met Lys Glu Asp Val Gly Arg Leu Leu Ile Gln Phe Lys Asn Glu Asn
1 5 10 15

Gly Glu Gly Leu Gly Thr Pro Phe Asp Val Pro Leu Asp Ile Thr Pro
20 25 30

Asp Lys Leu Gln Leu Val Cys Asn Ala Leu Leu Gln Glu Glu Asp Pro
35 40 45

Val Pro Leu Ala Phe Phe Val Gln Asp Leu Glu Ile Val Thr Ser Leu
50 55 60

Asp Lys Thr Leu Glu Lys Gln Ser Val Glu Thr Glu Lys Val Ile Asp
65 70 75 80

Ile Ile Tyr Gln Pro Gln Ala Val Phe Lys Val Arg Ala Val Thr Arg
85 90 95

Cys Thr Ser Ser Leu Glu Gly His Thr Glu Ala Val Ile Ser Val Ala
100 105 110

Phe Ser Pro Thr Gly Lys Tyr Leu Ala Ser Gly Ser Gly Asp Thr Thr
115 120 125

Val Arg Phe Trp Asp Leu Ser Thr Glu Thr Pro His Phe Thr Ser Lys
130 135 140

Gly His Thr His Trp Val Leu Ser Ile Ala Trp Ser Pro Asp Gly Lys
145 150 155 160

Lys Leu Ala Ser Gly Cys Lys Asn Ser Gln Ile Phe Ile Trp Asp Pro
165 170 175

Ser Thr Gly Lys Gln Ile Gly Lys Pro Leu Thr Gly His Ser Lys Trp
180 185 190

Ile Thr Trp Leu Cys Trp Glu Pro Leu His Leu Asn Pro Glu Ser Arg
195 200 205

Tyr Leu Ala Ser Ala Ser Lys Asp Cys Thr Ile Arg Ile Trp Asp Thr
210 215 220

Val	Met	Gly	Gln	Cys	Gln	Lys	Ile	Leu	Thr	Ser	His	Thr	Gln	Ser	Val
225					230					235					240

Thr Ala Val Lys Trp Gly Gly Asp Gly Leu Leu Tyr Ser Ser Ser Gln
245 250 255

Asp Arg Thr Ile Lys Ala Trp Arg Ala Gln Asp Gly Val Leu Cys Arg
260 265 270

Thr Leu Gln Gly His Ala His Trp Val Asn Thr Met Ala Leu Ser Thr
 275 280 285
 Asp Tyr Val Leu Arg Lys Gly Ala Phe Asn Pro Ala Asp Ala Ser Val
 290 295 300
 Asn Pro Gln Asp Met Ser Gly Ser Leu Glu Val Leu Lys Glu Lys Ala
 305 310 315 320
 Leu Lys Arg Ser Asn Glu Val Arg Gly Gln Gly Pro Glu Arg Leu Val
 325 330 335
 Ser Gly Ser Glu Asp Phe Thr Leu Phe Leu Trp Ala Pro Ala Glu Glu
 340 345 350
 Lys Lys Pro Leu Gln Arg Met Thr Gly His Gln Ala Leu Ile Asn Glu
 355 360 365
 Val Leu Phe Ser Pro Asp Thr Arg Ile Ile Ala Ser Ala Ser Phe Asp
 370 375 380
 Lys Ser Ile Lys Leu Trp Asp Gly Lys Thr Gly Lys Phe Leu Thr Ser
 385 390 395 400
 Leu Arg Gly His Val Ser Ala Val Tyr Gln Ile Ala Trp Ser Ala Asp
 405 410 415
 Ser Arg Leu Leu Val Ser Gly Ser Ser Asp Ser Thr Leu Lys Val Trp
 420 425 430
 Asp Ser Lys Thr Lys Lys Leu Leu Ile Asp Leu Pro Gly His Ala Asp
 435 440 445
 Glu Val Tyr Ser Val Asp Trp Ser Pro Asp Gly Gln Arg Val Ala Ser
 450 455 460
 Gly Gly Lys Asp Lys Cys Leu Arg Ile Trp Arg Lys
 465 470 475

<210> 8

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: HA epitope

<400> 8

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala
 1 5

<210> 9

<211> 149

<212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

<400> 9
 cggatccaaa aaatgtatcc ctatgacgtc cccgattatg cctaccctta cgatgtacct 60
 gactacgcgt atccgtacga cgttccggac tatgctcagg agacggacac ggagcaagag 120
 gccacgccac atacgataca ggcgcgcca 149

<210> 10
 <211> 23
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

<400> 10
 taaacgaggc gcgcctatcg tat 23

<210> 11
 <211> 32
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

<220>
 <221> modified_base
 <222> (12)
 <223> i

<220>
 <221> modified_base
 <222> (21)
 <223> i

<220>
 <221> modified_base
 <222> (24)
 <223> i

<220>
 <221> modified_base
 <222> (27)
 <223> i

<400> 11
 cgcagaattc cnttygaygt nccngtngay at 32

<210> 12
 <211> 32
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

<220>
 <221> modified_base
 <222> (15)
 <223> i

<220>
 <221> modified_base
 <222> (24)
 <223> i

<400> 12
 ggtgctcgag cytgnggytg rtanatdatr tc

32

<210> 13
 <211> 8
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Conserved
 peptide

<400> 13
 Pro Phe Asp Val Pro Val Asp Ile
 1 5

<210> 14
 <211> 7
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Conserved
 peptide

<400> 14
 Asp Ile Ile Tyr Gln Pro Gln
 1 5

<210> 15
 <211> 21
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: Primer

21

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<210> 16
<211> 21
<212> DNA
<213> Artificial Sequence
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<220>
<223> Description of Artificial Sequence: Primer

21